THE JOURNAL

OF

BIOLOGICAL CHEMISTRY

FOUNDED BY CHRISTIAN A. HERTER AND SUSTAINED IN PART BY THE CHRISTIAN A. HERTER MEMORIAL FUND

EDITED FOR THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS

VOLUME 159

BALTIMORE

1945

PUBLISHED AT YALE UNIVERSITY FOR
THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, INC.
WAVERLY PRESS, Inc.
Baltimore 2, U. S. A.

CONTENTS OF VOLUME 159

No. 1, June, 1945

Letters to the Editors

No. 2, JULY, 1945

Letters to the Editors

No. 3, AUGUST, 1945

Letters to the Editors

THE VISIBLE ABSORPTION BAND OF REDUCED LUCIFERIN

By AURIN M. CHASE

(*From the Physiological Laboratory, Princeton University, Princeton*)

(Received for publication, February 9, 1945)

The visible absorption spectrum of *Cypridina* luciferin (the substrate in a bioluminescent reaction (Harvey (5))), carried through two cycles of purification (method of Anderson (1)), has been measured in phosphate buffer of pH 6.8 (Chase (3)). These measurements were made with the Hardy recording spectrophotometer (Hardy (4)), which permits the tracing of an absorption spectrum within 5 minutes after the dry luciferin was dissolved. As Anderson (2) has shown, luciferin is reversibly oxidized by air to a compound which will not react in the ordinary way with the enzyme, luciferase, to give light. The measurements of the absorption spectrum by Chase (3) showed initially a band at about 435 mμ, which was replaced, within 20 minutes exposure to air, by one at about 465 mμ. The latter then disappeared during some 6 hours of additional exposure to air, leaving the solution practically colorless.

These spectral absorption data were interpreted by Chase (3) as showing that the 435 mμ band represents reduced luciferin;[1] *i.e.*, before any reaction with dissolved oxygen in the solvent has occurred. The band would therefore be an important property of the luciferin molecule, especially in conjunction with the subsequent changes in the absorption spectrum. It has become apparent, however, that an alternative interpretation of these data may be possible. The measurements described above were made on a solution which contained dissolved oxygen. It might therefore be that the band at 435 mμ does not represent reduced luciferin but, rather, that reduced luciferin is colorless and the band appears during the short interval when the freshly dissolved luciferin is in contact with oxygen in the solvent before the initial absorption spectrum is measured. It is necessary, consequently, to determine which of these two interpretations is correct.

Examination of the absorption spectrum of luciferin, freed from color impurity by two cycles of purification and dissolved and measured in complete absence of oxygen, would settle this point, since under such conditions there is no opportunity for reaction with oxygen and the absorption spectrum would be quite stable. Unfortunately, no such data exist, since only luciferin from a *single* cycle of purification has been measured in this way (3).

[1] The term "reduced luciferin," as used here, means luciferin that has not reacted spontaneously with oxygen.

However, these latter data can be made to yield the desired information, without the necessity for a specific experiment (which would require a relatively large amount of the limited supply of *Cypridina* material). Although the yellow impurity remaining after a single cycle of purification largely obscures specific visible absorption by luciferin itself, the changes in the absorption spectrum of an aerobic solution during exposure to air represent only luciferin and the absorption due to impurity is stable (3). Therefore, the spectrum of such a solution after prolonged exposure to air (when the luciferin has become colorless and the remaining color represents only impurity) can be subtracted from the initial spectrum of the same solution (measured as soon as possible after the luciferin was dissolved) and the "difference spectrum" should be that of the luciferin alone. As will presently be shown, the initial spectrum of the luciferin solution containing dissolved oxygen is the same as the spectrum of the luciferin solution prepared and measured in complete *absence* of oxygen. Consequently, both these solutions yield the same "difference spectrum" (representing reduced luciferin, freed from stable colored impurity), which turns out to have an absorption band at 435 mμ.

Analysis of Data, and Conclusions

Analysis of the data is somewhat complicated, since the luciferin solutions compared had been measured under rather different experimental conditions. Both contained luciferin from the same stock, having been carried through a single cycle of purification, but, while qualitatively the same, they differed in concentration. Furthermore, the anaerobic solution was measured in a 50 mm. absorption cell with nothing in the comparison beam of the spectrophotometer, whereas the aerobic solution was in a 10 mm. cell and the spectrophotometer's comparison beam passed through an identical cell containing distilled water. In the former case, therefore, the cell's reflection and slight selective absorption contributed to the spectrum measured.[2] The spectrum of the 50 mm. cell containing water has subsequently been obtained[3] with the same instrument used for the other measurements, and the values of $-\log T$ have been subtracted from the corresponding ones for the anaerobic luciferin solution. This gives an absorption spectrum uninfluenced by reflection and absorption of the cell and comparable to the initial spectrum of the freshly prepared aerobic solution.

[2] The point of interest when this spectrum was measured was not specific absorption but, rather, whether or not the color of the solution was stable in the absence of oxygen.

[3] Dr. A. C. Hardy and Dr. S. Q. Duntley, of the Massachusetts Institute of Technology Color Measurement Laboratory, very kindly supplied these measurements.

Table I contains the values for the anaerobic luciferin solution minus the values for the cell, as well as those for the solution which was measured as soon as possible after the dry luciferin was dissolved in the aerobic solvent. Since these two sets of values involve differences only of luciferin concen-

TABLE I

—Log T Values Used in Construction of Fig. 1

Wave-length	Anaerobic solution, corrected for cell	Initial measurement, aerobic solution	Final measurement, aerobic solution
$m\mu$			
400	0.478	0.347	0.215
420	0.478	0.347	0.097
440	0.424	0.314	0.060
460	0.301	0.229	0.043
480	0.169	0.131	0.034
500	0.105	0.078	0.024
520	0.089	0.060	0.018

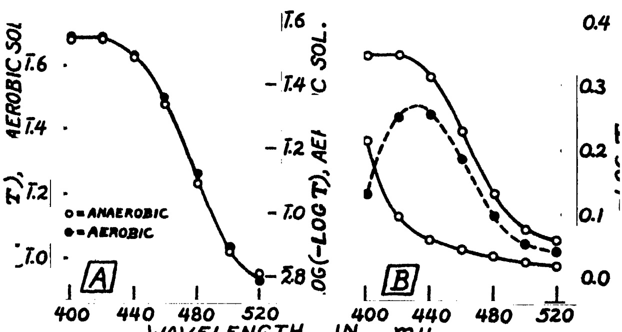


FIG. 1. (*A*) Values of log (—log *T*) from an aerobic luciferin solution measured as soon as possible after the luciferin was dissolved, and values from an anaerobic luciferin solution. The two curves have been shifted on the vertical axis to coincide, showing that the absorption spectra of the two solutions are qualitatively identical. (*B*) The uppermost curve represents the spectrum of the luciferin solution measured as soon as possible after dissolving the luciferin in an aerobic solvent. The lowest curve is the spectrum of the same solution after prolonged exposure to air, when no further change will occur. The middle curve is the difference between these two spectra, and consequently represents the luciferin itself. It shows a maximum at about 435 mμ.

tration and of optical depth of the absorption cells, the two spectra can easily be compared for qualitative similarity by plotting as log (—log *T*). Concentration differences, both real and apparent, cancel out when the curves are plotted in this way. If the two curves can be made to coincide

by shifting one of them on the log (−log T) axis, the two solutions, anaerobic and aerobic, are identical in spectral absorption.

Fig. 1, *A* shows the values of log (−log T) plotted against wave-length in mμ, and shifted on the vertical axis to coincide. It is quite apparent that there is no significant difference between the absorption spectra of the two solutions.

Having established the identity of the spectra of the oxygen-free solution and the freshly prepared aerobic solution, we must determine whether or not they show the 435 mμ absorption band when corrected for absorption by stable, colored impurity; this was done by subtracting from the initial spectrum of the aerobic, unstable solution its final spectrum after prolonged exposure to air. The necessary numerical values are presented in Table I. The initial, final, and "difference" spectra are plotted in Fig. 1, *B*. The difference spectrum clearly shows a maximum at about 435 mμ, just as is found from direct measurement of freshly prepared aerobic solutions of luciferin that has been carried through two cycles of purification so as to remove most of the stable, colored impurity which remains after a single cycle.

Since the absorption spectrum of the anaerobic luciferin solution has been shown to be identical with that of the aerobic solution immediately after the luciferin is dissolved, the same absorption maximum, at 435 mμ, must exist when the luciferin is dissolved and measured in complete absence of oxygen as when oxygen is present in the solvent. This absorption band must, therefore, be a property of reduced luciferin.

SUMMARY

By an analysis of spectrophotometric data on *Cypridina* luciferin solutions it is shown that the absorption band at 435 mμ which has been observed for freshly prepared aerobic luciferin solutions is not due to initial reaction of the luciferin with oxygen dissolved in the solvent. The band is a property of reduced[1] luciferin.

BIBLIOGRAPHY

1. Anderson, R. S., *J. Gen. Physiol.*, **19**, 301 (1935).
2. Anderson, R. S., *J. Cell. and Comp. Physiol.*, **8**, 261 (1936).
3. Chase, A. M., *J. Biol. Chem.*, **150**, 433 (1943).
4. Hardy, A. C., *J. Optical Soc. America*, **25**, 305 (1935).
5. Harvey, E. N., Annual review of biochemistry, Stanford University, **10**, 531 (1941).

THE EFFECT OF CHOLINE INTAKE AND ENVIRONMENTAL TEMPERATURE ON THE EXCRETION OF CHOLINE FROM THE HUMAN BODY*

By B. CONNOR JOHNSON, T. S. HAMILTON, and H. H. MITCHELL

(From the Division of Animal Nutrition, University of Illinois, Urbana)

(Received for publication, March 5, 1945)

Choline was shown to be essential for the rat by Best and Huntsman (1) and for the dog by Schaefer, McKibbin, and Elvehjem (2). Mills (3) has reported an increased choline requirement for rats kept under high environmental temperatures. However, the experiments were not sufficiently well controlled, nor adequately analyzed statistically, to support an unequivocal conclusion. Horowitz and Beadle (4) have developed a microbiological assay method for choline sensitive enough to determine the amounts present in sweat and urine, thus enabling us to study the effect of an increased environmental temperature upon the excretion of choline by the adult human. Choline balances were carried out under both comfortable and hot environments.

EXPERIMENTAL

Four adult male subjects, age 21 to 28 years, were used in this study. These subjects remained in a chamber in which the temperature and humidity were accurately controlled for 8 hours per day for 5 day periods. During the first experimental period "comfortable" conditions of 29° and 50 per cent relative humidity were maintained. During the second experimental period "hot moist" conditions of 37.7° and 70 per cent relative humidity were maintained. The subjects were fed a constant diet during a preliminary period as well as the experimental periods during which collections were made.

Total skin excretions (body washings and sweat during the hot week; body washings during the comfortable week) were collected for the 8 hour period in the chamber each day. Collections of the total urinary and fecal excretions were made for the 5 day experimental periods, the feces being separated by means of fecal markers (charcoal). Aliquots of the food were taken each day. The food was preserved by freezing, the feces by drying, and the body washings, sweat, and urine with hydrochloric acid.

* The data reported in this paper were obtained in the course of a project under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Illinois.

Assay Procedures—The samples were analyzed for choline by the microbiological procedure of Horowitz and Beadle[1] (4), 1 per cent asparagine being added to their basal medium to give somewhat better growth.

The choline content of the body washings and sweat was so low that a concentration procedure was necessary. It was found that the permutit adsorption column in the original procedure could be used satisfactorily for this purpose. 100 to 200 cc. of body washings (or diluted sweat) were adjusted to pH 6.5 and put through columns as described by Horowitz (4). Each column was then eluted with 10 cc. of 5 per cent NaCl and the two

TABLE I

Intake and Output of Choline under Comfortable and under Hot Moist Conditions

Week No.	Condition	Subject	Choline intake	Choline output				Choline balance*	Per cent of intake excreted			
				Feces	Urine	Skin	Total		Feces	Urine	Skin	Total
			mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*				
34th	Comfortable	C	623.8	3.32	6.08	0.023	9.42	614.4	0.53	0.97	0.0036	1.51
		D	856.8	4.52	7.85	0.073	12.44	844.4	0.53	0.92	0.0085	1.45
		E	673.2	3.20	4.28	0.023	7.50	665.7	0.48	0.64	0.0034	1.11
		F	763.6	1.65	3.94	0.015	5.60	758.0	0.22	0.52	0.0019	0.73
Average			729.4	3.17	5.54	0.034	8.74	720.6	0.44	0.76	0.0044	1.20
35th	Hot moist	C	694.8	2.59	3.43	0.067	6.09	688.7	0.37	0.49	0.0096	0.88
		D	898.7	5.31	6.83	0.101	12.24	886.5	0.59	0.76	0.0112	1.36
		E	691.0	4.72	4.13	0.063	8.91	682.1	0.68	0.60	0.0091	1.29
		F	690.8	2.48	4.86	0.075	7.42	683.4	0.36	0.70	0.0108	1.07
Average			743.8	3.78	4.81	0.076	8.66	735.2	0.50	0.64	0.0102	1.15

* This would include any choline retained as well as the choline catabolized in the tissues.

eluates combined and analyzed as usual. Recoveries of added choline of 98 per cent and 100 per cent were obtained.

All the choline in sweat and in urine was found to be present in the free form, no higher values being obtained after acid hydrolysis. The same finding has been reported for urine by Luecke and Pearson (5).

Results

The choline balances of the four subjects are given in Table I. From this table it can be seen that only a very small percentage (0.73 to 1.51) of the choline intake is excreted. There is no significant difference in the

[1] We are indebted to Dr. Horowitz for a culture of *Neurospora crassa cholineless* No. 34486.

total excretion of choline under comfortable as compared to hot moist conditions. However, there is a significant increase in the skin excretion of choline under the hot moist conditions. This is balanced by a concomitant decrease in urinary output of choline.

Table II gives the choline concentration data for undiluted sweat obtained while subjects were on a normal diet and on the same diet plus 6 gm. of added choline. In all cases except Subject D there is a definite increase in the concentration of choline in the sweat after choline dosage.

DISCUSSION

Only a very small proportion of the choline intake in this study was found to be excreted as unchanged choline. Expressed as a per cent of the intake, the losses of choline were 0.44, 0.76, and 0.0044 in feces, urine, and skin excretions, respectively, during the comfortable period; and 0.50, 0.64,

TABLE II

Choline Content of Undiluted Sweat, with and without Choline Dosage

Subject	Choline concentration (no additional choline administered)	Choline concentration (6 gm. choline chloride in addition to diet*)
	γ per 100 cc.	*γ per 100 cc.*
C	2.7	7.5
D	15.3	8.5
E	6.1	8.8
F	4.4	9.2
Average..	7.1	8 5

* Choline given in addition to that in the diet.

and 0.0102 in feces, urine, and skin excretions, respectively, during the hot moist period. Apparently 98.5 to 99 per cent of the choline ingested was metabolized in the body.

Of the small amount of choline excreted 63.5 per cent was excreted in the urine, 36.2 per cent in the feces, and only 0.3 per cent through the sweat during the "comfortable" period. During the "hot moist" period the comparable figures were 55.5 per cent, 43.6 per cent, and 0.9 per cent.

A "hot moist" environment was found to have no effect on the loss of choline from the body. This does not necessarily imply that there is not an increased requirement in the body.

SUMMARY

1. Choline balances were determined on four adult male subjects on an adequate diet under "comfortable" and under "hot moist" environmental conditions.

2. Only 0.7 to 1.5 per cent of the choline intake was excreted as choline.

3. No increase in total choline excretion was found under "hot moist" conditions.

4. Choline was found in undiluted sweat in amounts of 2.7 to 15.30 γ of choline per 100 cc.

BIBLIOGRAPHY

1. Best, C. H., and Huntsman, M. E., *J. Physiol.*, **75**, 405 (1932).
2. Schaefer, A. E., McKibbin, J. M., and Elvehjem, C. A., *Proc. Soc. Exp. Biol. and Med.*, **47**, 365 (1941).
3. Mills, C. A., *Arch. Biochem.*, **1**, 73 (1942); **2**, 159 (1943); *Proc. Soc. Exp. Biol. and Med.*, **54**, 265 (1943).
4. Horowitz, N. H., and Beadle, G. W., *J. Biol. Chem.*, **150**, 325 (1943).
5. Luecke, R. W., and Pearson, P. B., *J. Biol. Chem.*, **153**, 259 (1944).

A COLORIMETRIC METHOD FOR THE MICRODETERMINATION OF α-ALANINE IN BLOOD*

BY BENJAMIN ALEXANDER AND ARNOLD M. SELIGMAN

(From the Medical and Surgical Research Laboratories, Beth Israel Hospital, and the Departments of Medicine and Surgery, Harvard Medical School, Boston)

(Received for publication, February 19, 1945)

In connection with investigation of intermediary metabolism the need arose for a specific micromethod for the determination of α-alanine in blood. Methods already proposed for the measurement of alanine were not only too insensitive for use on blood or other biological material in which the amount of alanine is very small but also lacked simplicity and specificity for general use.

Among these methods is that of Fürth, Scholl, and Herrmann (1), who converted alanine to lactic acid by means of nitrous acid. Using 0.1 to 0.5 mg. of alanine, they determined the lactic acid by the technique of Friedemann, Cotonio, and Shaffer (2). More recently Bloch *et al.* (3) measured the lactic acid thus produced by converting it into acetaldehyde by oxidation. The acetaldehyde was measured with *p*-hydroxybiphenyl. Both methods (1, 3) require not only a predetermination of lactic acid and acetaldehyde in those biological materials in which these substances may occur, but also removal or measurement of other agents readily yielding these substances. Thus the method of Bloch *et al.* (3) requires a simultaneous determination of threonine, since it too yields acetaldehyde on oxidation.

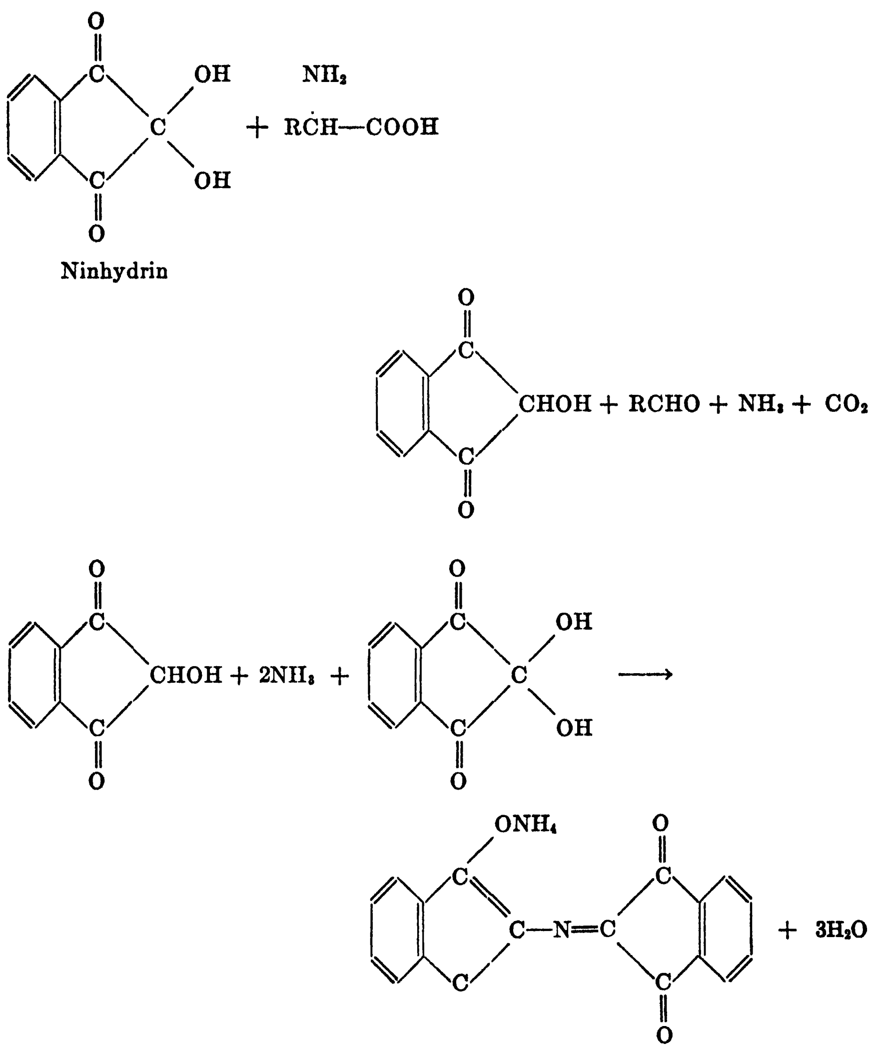
More recently Bergmann (4) described a gravimetric method in which dioxpyridic acid is employed to precipitate the alanine. This method is unsatisfactory for micro measurement. Furthermore incomplete recoveries of alanine by this technique necessitate the application of correction factors calculated from recoveries of added alanine.

During the course of this investigation we learned that Virtanen *et al.* (5) in 1940 published a method for alanine determination based upon the same reaction which we employ. For reasons which will appear later their method is not only less specific, but also less sensitive than the one we propose.

The reaction of ninhydrin (triketohydrindene hydrate) with amino acids to give carbon dioxide and the aldehydes of the decarboxylated and de-

* This investigation has been aided by a grant from the Josiah Macy, Jr., Foundation. The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

aminated amino acids has been well elucidated (6–9). According to Ruhemann (7), the reaction proceeds according to the accompanying formula. Calvery (10) stated that measurement of the color resulting from further



Diketohydrindylidendiketohydrindamine
(blue color)

reaction of the reduced ninhydrin with another molecule of ninhydrin and ammonia could not be used for measuring total amino acids because of differences in tinctorial value of the color from different amino acids. Van

Slyke and Dillon (11) and Mason (12) have measured the carbon dioxide evolved in the course of this reaction from which the total amino acid content was calculated. The former authors found that the reaction was specific for carboxyl groups of amino acids, 1 mole of CO_2 being liberated from 1 mole of amino acid within 3 minutes. The dicarboxylic acids, aspartic and glutamic, yielded 2 moles of CO_2 but in the latter the 2nd mole of CO_2 was liberated much more slowly than the 1st one. Under practically the same conditions, Mason (12) could recover only 75 to 90 per cent of the theoretical CO_2 from alanine. Both authors agree that the reaction is specific for compounds having primary amino groups in the α position to carboxyl groups; peptides and β- and ϵ-amino acids do not yield aldehydes and carbon dioxide.

Our method for the determination of alanine is based upon its conversion by ninhydrin to acetaldehyde, which is separated from the reaction mixture and measured from the color produced when it is allowed to react with *p*-hydroxybiphenyl. By the method outlined below alanine can be recovered quantitatively from blood and from hydrolysates of polypeptides (Table I) with good duplicability (Table II).

Procedure

Reagents—

Ninhydrin solution, 1.0 gm. in 100 ml. of H_2O (triketohydrindene hydrate, Eastman).

Phosphate buffer, pH 5.5 (glass electrode). 3.5 gm. of tripotassium phosphate (K_3PO_4) are added to 100 ml. of a 20 per cent solution of primary potassium phosphate (KH_2PO_4).

10 per cent sodium tungstate solution.

0.66 N sulfuric acid.

1.0 per cent sodium bisulfite solution.

4.0 per cent copper sulfate solution.

1.5 per cent solution of *p*-hydroxybiphenyl in 0.5 per cent sodium hydroxide.

C.P. sulfuric acid, concentrated, sp. gr. 1.84.

In the flask of a specially constructed aeration still (Fig. 1) are placed 5.0 ml. of a protein-free blood filtrate (1:10 dilution) prepared according to Stotz (13) as follows: 1.0 ml. of blood was added to 7.0 ml. of water and 1.0 ml. of sodium tungstate solution was then added, followed by 1.0 ml. of 0.66 N sulfuric acid. After standing, the precipitate was removed by filtration or centrifugation. To 5.0 ml. of this filtrate are added 1.0 ml. of ninhydrin solution, 2 ml. of the phosphate buffer, and a glass bead. The flask is then attached to the aeration still on the receiving end of which is connected a test-tube containing 8.0 ml. of sodium bisulfite solu-

tion. This tube is kept cold with an ice bath. The apparatus is then connected to gentle suction. The reaction mixture is brought to a boil

TABLE I

Recovery of Alanine

Material	Alanine present	Added alanine	Recovery of added alanine		Recovery of total expected alanine	Method
	γ	γ	γ	*per cent*	*per cent*	
Human blood	23.8	50.0	52.9	105.8	96.0	Aeration
" "	23.8	100.0	94.6	94.6	95.0	"
" "	23.8	125.0	117.2	87.0	94.0	"
" "	23.8	150.0	135.4	90.3	90.5	"
Dog blood*	45.9	13.3	8.2	62.0	90.7	"
" " *	45.9	26.6	24.6	92.0	97.0	"
" " *	45.9	40.0	34.6	87.0	94.0	"
" " *	45.9	53.2	52.6	98.0	99.4	"
" " *	45.9	66.6	65.9	99.0	99.4	"
" " protein-free filtrate	25.0	250.0	262.5	104.8	105.0	Sealed tube
Gramicidin hydrolysate†	40.0	50.0	42.6	85.4	91.8	" "
Amino acid mixture	12.6	50.0	43.0	86.0	88.8	" "

* Variable amounts of glycine as well as alanine were added to each specimen of dog blood.

† We are indebted to Dr. Halvor Christensen of the Harvard Medical School for the sample of gramicidin hydrolysate.

TABLE II

Duplicability of Alanine Determination

Material	Alanine
	γ
Blood	141
	133
" filtrate	23.4
	23.0
	23.4
	27.5
Alanine standard	110
	112
	103
" "	113
	116

and refluxed for 75 minutes while a steady stream of air is being swept through the apparatus. The receiving test-tube is then removed, the

bisulfite solution containing the acetaldehyde is transferred quantitatively to a graduated centrifuge tube, and, with the washings, is made to a total volume of 10.0 ml.

1 ml. is analyzed by Barker and Summerson's method for lactic acid (14), as modified by both Stotz (13) and us as follows: 1 drop of 4.0 per cent copper sulfate is added, followed by 6.0 ml. of concentrated sulfuric acid from a burette. The test-tube is cooled to 37° by being placed in a

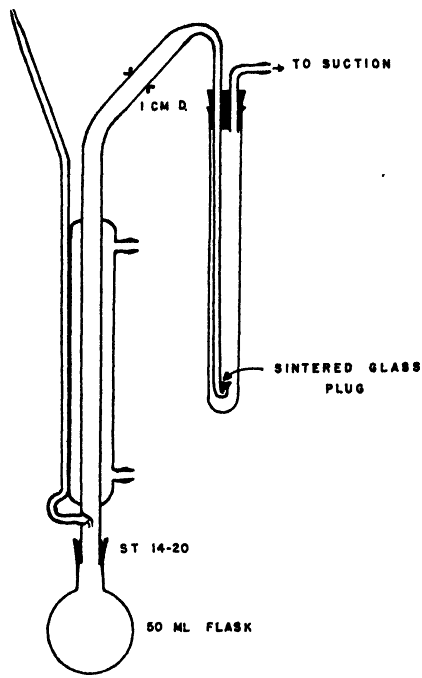


FIG. 1. Apparatus for aeration of acetaldehyde from refluxing reaction mixture. The air inlet tube is placed in contact with the water-cooled condenser to condense any steam that may back up during bumping. The sintered glass plug may be replaced by a capillary opening.

water bath, and 2 drops (0.1 ml.) of the *p*-hydroxybiphenyl solution are added. The mixture is agitated vigorously and the test-tube put in the incubator at 37.5° for 30 minutes. The tube is placed in a boiling water bath for 1.5 minutes and is then cooled to room temperature in a water bath. The intensity of the violet color which develops is compared with that obtained from a standard alanine or zinc lactate (14) solution in the Klett photoelectric colorimeter with Filter 540.

Although the molecular weights of alanine (89) and lactic acid (90) are

nearly identical, calibration curves prepared for the determination of lactic acid (according to the method of Barker and Summerson (14)) cannot be used, because treatment with copper-lime reduces the density of color. A separate calibration curve is therefore prepared either from a zinc lactate solution directly, the copper-lime treatment (14) being omitted, or from a pure alanine solution, according to the procedure outlined above.

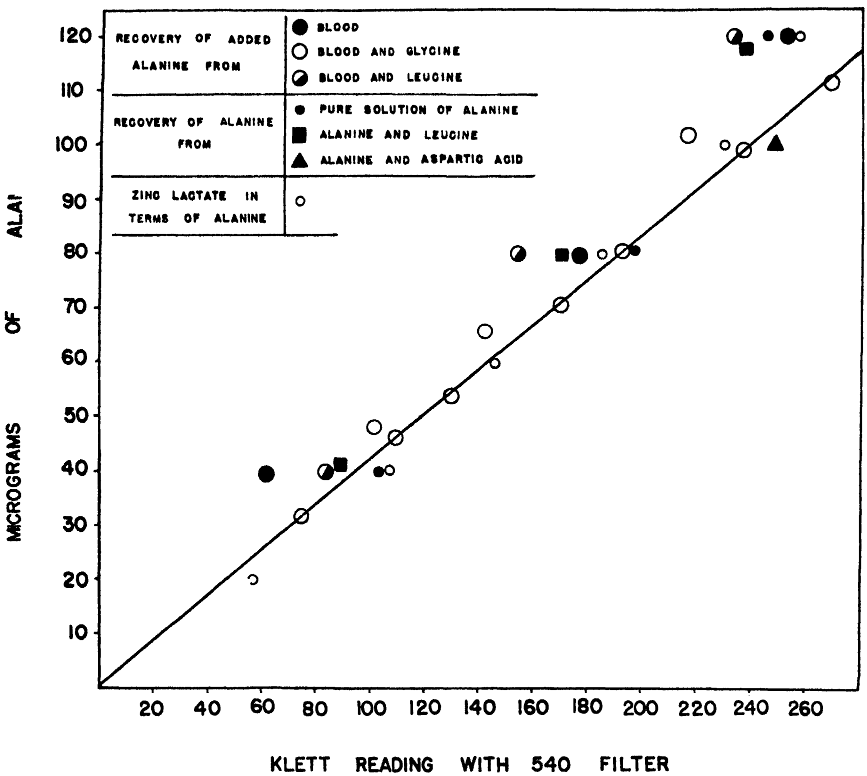


FIG. 2. Relationship between the amount of alanine converted and intensity of color obtained. The line was drawn by inspection through values obtained for alanine which had been added to blood together with varied amounts of glycine.

It is evident (Fig. 2) that the relationship between the concentration of alanine and the intensity of color is linear between 20 and 100 γ. The sensitivity of the method as described is limited approximately to 20 γ of alanine in 5.0 ml. of blood filtrate (40 γ per ml. of whole blood). For blood levels lower than this the sensitivity of the method may be increased by (1) making a more concentrated blood filtrate, (2) using a larger volume of blood filtrate, (3) aerating the acetaldehyde into a smaller volume

of bisulfite solution, and (4) redistillation of the bisulfite acetaldehyde solution into a smaller volume of bisulfite according to the method of Stotz (13). By one or another of these modifications as little as 0.5 mg. per cent of alanine in blood can be measured.

By the method described no acetaldehyde or significant color could be obtained from any of the following substances, lactic acid, pyruvic acid, alanylglycine, benzoylalanine, β-alanine, threonine, serine, phenylalanine, glutamic acid, tryptophane, cystine, cysteine, valine, ornithine, lysine, α-amino-*n*-butyric acid, methionine, arginine, isoleucine, tyrosine, diiodotyrosine, α-aminoisobutyric acid, proline, or hydroxyproline.

DISCUSSION

Decarboxylation and Deamination of Alanine by Ninhydrin—The explanation for Mason's (12) observation that incomplete decarboxylation of alanine occurred when it was treated with ninhydrin was found by us

TABLE III

Effect of pH on Conversion of Aspartic Acid to Acetaldehyde by Ninhydrin Reaction

pH	Recovery of aspartic acid from 500 γ		Procedure
	γ	*per cent*	
4.0	66	13.2	Autoclaved in sealed tube for 10 min.
4.0	67	13.4	Aeration from refluxing mixture for 1¼ hrs.
4.5	43	9.0	" " " " " 1½ "
5.0	40	8.0	" " " " " 1½ "
5.5	20	4.0	" " " " " 1½ "

to be due to the slowness of the reaction. The theoretical yield of acetaldehyde from alanine (0.02 to 0.2 mg.) could be obtained with 10 mg. of ninhydrin only after boiling the reaction mixture for at least 1 hour or by heating it in a sealed tube in an autoclave at 125° for 10 minutes. Mason, on the other hand, ran his reaction at boiling temperature for only 3 minutes with 24 times the concentration of ninhydrin we used. Although increase of the concentration of ninhydrin does increase the rate of the reaction, the conditions used by Mason did not result in complete reaction, as indicated by the evolution of CO_2.

Interference from Aspartic Acid—If aspartic acid gives 2 moles of CO_2 (12) during its reaction with ninhydrin, acetaldehyde must result. The finding of Virtanen *et al.* (5) that sufficient quantities of acetaldehyde were formed from aspartic acid to interfere with the determination of alanine if the reaction was conducted at pH 4.0 was confirmed. The yield of acetaldehyde from aspartic acid can, however, be influenced by the pH at which the reaction takes place (Table III). When the reaction was

carried out at pH 5.5 to 5.6, 4 per cent of the aspartic acid was determined as alanine, whereas no interference with alanine recovery was noted (aeration method).

Interference of Other Amino Acids—The measurement of acetaldehyde in a mixture of aldehydes resulting from the reaction of various amino acids with ninhydrin depends first upon the separation of the volatile acetaldehyde from the mixture. Although volatile aldehydes other than acetaldehyde are produced from several amino acids, most of them do not react with *p*-hydroxybiphenyl to give a colored compound. This reaction, first described by Eegriwe (15) and subsequently used to measure lactic acid (14) and acetaldehyde (13), is highly, but unfortunately not completely, specific for acetaldehyde. We have found that besides acetaldehyde, formaldehyde (obtained from glycine), isovaleraldehyde (from leucine), *n*-valeraldehyde (from norleucine), and *n*-butyraldehyde (from norvaline) react with *p*-hydroxybiphenyl to yield colored derivatives. In contradistinction to acetaldehyde which gives a violet color, formaldehyde gives a blue-green color (14) and isovaleraldehyde and *n*-valeraldehyde give a rose-pink color. *n*-Butyraldehyde, however, gives a violet color, almost indistinguishable from that obtained with acetaldehyde.

Although Virtanen *et al.* (5) claim that no volatile aldehyde was produced from a reaction mixture containing glycine and ninhydrin, it was found that formaldehyde is not only obtained quantitatively,[1] but also is sufficiently volatile with steam to interfere with the determination of alanine. This difficulty could be circumvented by separating the acetaldehyde from formaldehyde by prolonged aeration of the refluxing reaction mixture. Such a procedure has been employed by Shinn and Nicolet (16) in their method for determining threonine. The acetaldehyde which was received into bisulfite was determined by the method of Barker and Summerson (14) with slight modification.

In the absence of glycine and aspartic acid, a prolonged aeration may be substituted by direct distillation of the acetaldehyde according to the method of Stotz (13). Since the reaction between alanine and ninhydrin is relatively slow, sufficient time must be allowed for its completion. Heating at 100° for 1 hour or in the autoclave at 17 pounds pressure for 10 minutes in a sealed tube is sufficient if 10 mg. of ninhydrin are used. In either case the reaction must be run at a pH of 4.0, since it was found that at a higher pH substantial loss in acetaldehyde occurred. This would seem to indicate that at a pH higher than 4.0 acetaldehyde must be removed as fast as it is produced in order to obtain quantitative recoveries, because with the aeration method no loss was noted at pH 5.5.

[1] A method for measuring glycine based upon this reaction will appear in another publication.

Among other aldehydes which are aerated from a refluxing mixture of amino acids during reaction with ninhydrin is isovaleraldehyde produced from leucine. A cold water trap, interposed between the still and the receiving bisulfite solution, did not prevent the isovaleraldehyde from being carried over quantitatively into the bisulfite. The recovery by Virtanen *et al.* (5) of 94 to 101 per cent of alanine from a mixture of amino acids containing alanine and leucine cannot, therefore, be attributed to more efficient condensation by their still, since these authors used essentially the same technique of aeration as is reported here. More probably their apparently complete recovery of alanine is the result of an increment of isovaleraldehyde from leucine which offset the incomplete conversion of alanine to acetaldehyde because their reaction was allowed to run for 30 minutes only. These authors measured aldehydes by their

TABLE IV

Effect of Temperature on Reaction between p-Hydroxybiphenyl and Molecularly Equivalent Quantities of Various Aldehydes As Indicated by Klett Readings with Filter 540

Reaction temperature	Acetaldehyde from alanine	*n*-Valeraldehyde from norleucine	*n*-Butyraldehyde		Isovaleraldehyde	
			From norvaline	Synthetic	From leucine*	Synthetic
°C.						
5					179	156
20–25	576	142	179	167	121	126
37	579	83	114	112	77	39

* This leucine sample may have contained some alanine which could account for the discrepancy at 37° between the values obtained for leucine and synthetic isovaleraldehyde.

bisulfite-binding power, a method which is not specific for any particular aldehyde.

Effect of Temperature on Interference from Leucine, Norleucine, and Norvaline—When leucine was allowed to react with ninhydrin, the resulting aldehyde gave considerable color with *p*-hydroxybiphenyl. About 5 parts of leucine gave the same intensity of color as 1 part of alanine. How much of this was due to leucine and how much to possible contamination of the sample with alanine was difficult to ascertain. It soon became evident that the degree of color developed from a constant amount of leucine varied with the temperature of the sulfuric acid mixture when the *p*-hydroxybiphenyl solution was added. The effect of increasing the temperature at which this reagent was allowed to react with the aldehyde was to decrease considerably the intensity of color produced (Table IV). Under the same conditions acetaldehyde from alanine gave the same

degree of color as was obtained at lower temperatures. The colors produced from three different samples (one synthetic) of leucine were not only greater than that produced from an equivalent amount of freshly redistilled isovaleraldehyde, but the colors also varied from one to another. Since the effect of increasing the temperature of the reaction with the sulfuric acid-*p*-hydroxybiphenyl mixture upon the color produced from all samples of leucine was less than on the color obtained with isovaleraldehyde, it would seem that our leucine samples were contaminated with alanine. An amount of isovaleraldehyde equivalent to 22 parts of leucine is required to give a color of the same intensity as 1 part of alanine. About 11 parts of our purest sample of leucine will do the same.

The interference from norleucine and norvaline was more difficult to circumvent. The corresponding aldehydes obtained from these amino acids in their reaction with ninhydrin are *n*-valeraldehyde and *n*-butyraldehyde respectively. About 10.5 parts of norleucine or 6.5 of norvaline are required to give a color of the same intensity as 1 part of alanine. Here, too, the question arises concerning possible contamination of these preparations of the amino acids with alanine.

The purity of our norleucine sample was not checked by comparing the color obtained from it with that produced by equivalent amounts of pure synthetic *n*-valeraldehyde. An increase of the temperature of the aldehyde-sulfuric acid mixture before the addition of the *p*-hydroxybiphenyl, however, had the same effect on the intensity of color as was obtained with isovaleraldehyde. By raising the temperature to 37° the interference from norleucine was reduced (Table IV). This interference should occur infrequently, since norleucine is a rare constituent of proteins (17).

Similarly the color obtained from both synthetic *n*-butyraldehyde and the aldehyde produced from the reaction of norvaline and ninhydrin was decreased by running the color reaction at a higher temperature (Table IV). Equivalents of *n*-butyraldehyde and norvaline were equally affected by this procedure and the colors developed from each were of the same intensity.

Alloxan, which according to Strecker (6) and Ruhemann (7) reacts similarly to ninhydrin with amino acids, did not react quantitatively with alanine (0.2 mg.). Only a slight increase in acetaldehyde production resulted from lowering the pH to 2 and increasing the concentration of alloxan 40 times.

SUMMARY

1. A method is described for the microdetermination of α-alanine in blood.

2. Of twenty-four other amino acids tested, twenty gave no interference; the interference of three was largely eliminated. Norvaline interfered at most to the extent that 6 parts were required to give the same color intensity as 1 part of alanine.

Acknowledgement is due Miss Gertrude Weinberger for technical assistance.

BIBLIOGRAPHY

1. Fürth, O., Scholl, R., and Herrmann, T. E., *Biochem. Z.*, **251,** 404 (1932).
2. Friedemann, T. E., Cotonio, M., and Shaffer, P. A., *J. Biol. Chem.*, **73,** 335 (1927).
3. Bloch, R. J., Bolling, D., and Webb, M., *Proc. Am. Soc. Biol. Chem.*, *J. Biol. Chem.*, **133,** p. xiv (1940).
4. Bergmann, M., *J. Biol. Chem.*, **122,** 569 (1937–38).
5. Virtanen, A., Laine, T., and Toivonen, T., *Z. physiol. Chem.*, **266,** 193 (1940).
6. Strecker, A., *Ann. Chem.*, **123,** 363 (1862).
7. Ruhemann, S., *J. Chem. Soc.*, **99,** 792 (1911).
8. Grassmann, W., and von Arnim, K., *Ann. Chem.*, **510,** 288 (1934).
9. Abderhalden, R., *Z. physiol. Chem.*, **252,** 81 (1935).
10. Calvery, H. O., in Schmidt, C. L. A., The chemistry of the amino acids and proteins, Springfield, 186 (1938).
11. Van Slyke, D. D., and Dillon, R. T., *Compt.-rend. trav. Lab. Carlsberg*, **22,** 480 (1938); *Proc. Soc. Exp. Biol. and Med.*, **34,** 362 (1938).
12. Mason, M., *Biochem. J.*, **32,** 719 (1938).
13. Stotz, E., *J. Biol. Chem.*, **148,** 585 (1943).
14. Barker, S. B., and Summerson, W. H., *J. Biol. Chem.*, **138,** 535 (1941).
15. Eegriwe, E., *Z. anal. Chem.*, **95,** 323 (1933).
16. Shinn, L. A., and Nicolet, B. H., *J. Biol. Chem.*, **138,** 91 (1941).
17. Karrer, P., Organic chemistry, New York, 260 (1938).

A SPECIFIC MICROMETHOD FOR THE DETERMINATION OF ACYL PHOSPHATES*

By FRITZ LIPMANN and L. CONSTANCE TUTTLE

(*From the Biochemical Research Laboratory, Massachusetts General Hospital, Boston*)

(Received for publication, February 14, 1945)

During the last few years it was observed that anhydrides of carboxylic and phosphoric acids appear as intermediates in metabolism. For one of these, acetyl phosphate, a method of determination had been worked out (1), based on a differential precipitation of calcium acetyl and inorganic phosphate with subsequent phosphate determination in the separate fractions. Such a procedure, founded on a more or less fortuitous behavior of the acetyl phosphate, lacked a desirable specificity for the characteristic anhydride group. In this communication, a method is described which utilizes the reaction of acyl phosphates with hydroxylamine. The acyl part of the acid anhydride is converted into hydroxamic acid.

$$R \cdot COOPO_3^{=} + H_2NOH \rightarrow R \cdot CONHOH + HOPO_3^{=}$$

The hydroxamic acid forms, with trivalent iron, a brightly purplish complex which in the past has been used for qualitative identification of acid derivatives after conversion into acyl chlorides (2), or of aldehydes (Angeli-Rimini test) after reaction with nitrohydroxylamine (*cf.* (3)). An adaptation of this reaction for quantitative determination was not found in the literature, but conditions were obtained relatively easily for measuring, quantitatively and specifically, acyl phosphates with the color reaction. At high concentrations acid amides react somewhat with hydroxylamine, but not at concentration levels expected to occur in physiological experiments. 0.25 micromole of acyl phosphate, or 8 γ of acyl phosphorus, is about the lower limit of the present method.

Following the description of the method, a few examples will be given of determination of acetyl and phosphoglyceryl phosphate when formed with bacterial and muscle preparations.

EXPERIMENTAL

Outline—Since acyl phosphates are unstable even at moderate acidity or alkalinity, it was desirable to postpone deproteinization until after conversion into hydroxamic acids had taken place. The hydroxamic acids are very stable compounds. The first step in this procedure, therefore, is the addition of an aliquot of the test solution to a slightly acid solution of hy-

* This research was supported by a grant from the Commonwealth Fund.

droxylamine. With a concentrated hydroxylamine solution less than 10 minutes at room temperature are sufficient to convert acyl phosphate quantitatively to hydroxamic acid.

After the conversion to hydroxamic acid is completed, protein is most conveniently removed by trichloroacetic acid precipitation. To develop the color, the sample is strongly acidified by addition of hydrochloric acid and, without removal of the protein precipitate, a 5 per cent solution of ferric chloride is added. Depending on the concentration of acyl phosphate, the color shade will be from orange-brown to purplish brown, due to the presence of the yellow color of the iron ion. The main absorption of the purple iron complex is between 540 and 480 mμ, at which ferric chloride does not absorb.

In the procedure, as described below, the volumes are adapted to the conditions of the Klett-Summerson photoelectric colorimeter. With this instrument the whole procedure may be carried out in the same colorimeter tube if the protein precipitate is small. After centrifuging, a moderate precipitate at the bottom of the tube does not disturb the measurement (4). If large protein precipitates are to be handled or the sample has to be clarified by filtration, a multiple of the stated volumes may, of course, be used. The relative concentrations of the reagents, however, have to remain constant.

Reagents—

Hydroxylamine solution. A 28 per cent solution of hydroxylamine hydrochloride (4 M) is nearly neutralized by addition of an equal volume of 14 per cent sodium hydroxide (3.5 M). The mixture has a pH of 6.4. This neutralized hydroxylamine solution is of limited stability and is best prepared daily from stock solution. The solution of hydroxylamine hydrochloride may be kept for longer periods, preferably in the cold.

Acetate buffer. A mixture of acetic acid and sodium acetate, both 0.1 M in 2:8 proportions, pH 5.4.

Hydrochloric acid. 1 volume of concentrated acid is diluted with 3 volumes of water.

Ferric chloride. A 5 per cent solution of ferric chloride, $6H_2O$, in 0.1 N hydrochloric acid.

Trichloroacetic acid. 12 per cent.

Procedure

0.5 to 1 ml. of test solution (the pH may vary from 5.5 to 7.5) is added to a mixture of 1 ml. of hydroxylamine solution and 1 ml. of acetate buffer. The volume is adjusted to 3 ml. and the mixture kept standing for 10 minutes at room temperature.

Thereafter, 1 ml. each of hydrochloric acid, trichloroacetic acid, and fer-

ric chloride solution is added in the indicated order. The protein precipitate is removed by centrifugation or filtration and the color measured in the Klett-Summerson instrument. For the Evelyn colorimeter all volumes are doubled. The Klett Filter 54 or the Evelyn Filter 540 is used (Fig. 1). Readings may be taken after 5 to 30 minutes standing. If the samples are kept longer, the color fades slowly.

The procedure may be interrupted for periods up to 20 hours, and probably longer, after addition of trichloroacetic acid without the hydrochloric acid. At that rather weak acidity, pH 6.1, the hydroxamic acid is stable,

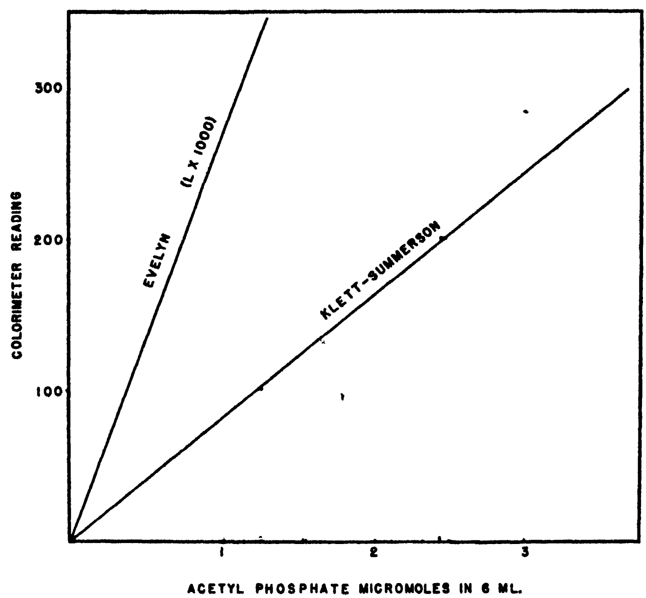


Fig. 1. Proportionality between color and acetyl phosphate concentration when measured with the Klett and with the Evelyn colorimeters.

but after standing with the strong hydrochloric acid a slow decrease of color was observed.

Specificity—As described, the method may be considered specific for carboxylic acid anhydrides. In concentrated solution and on heating, acid amides and, to a lesser degree, esters will react with hydroxylamine in a similar manner. Aldehydes yield hydroxamic acids only when heated with nitrohydroxylamine or N-benzenesulfonylhydroxylamine (*cf.* (3)). They do not require consideration under our conditions. Relative data for a number of compounds are assembled in Table I. It appears that acetyl, propionyl, and butyryl phosphate (1) produce practically equal color, mole per mole. As a representative for water-stable purely organic anhydrides,

succinic anhydride was tried. The color intensity was somewhat less with succinic anhydride than with acetyl phosphate. The data of Table I show furthermore that at physiological concentrations acid amides and esters may be neglected under our conditions.

Standard Solutions—In our experiments solutions of acetyl phosphate were used, derived from pure silver acetyl phosphate. Its preparation has been described previously (1). A very satisfactory standard solution is obtained, however, with the commercial succinic anhydride preparation (Eastman). For this purpose the compound, which is only slightly soluble in water, is immediately converted into the soluble monohydroxamic acid by dissolving in a concentrated hydroxylamine solution. We dissolve, in a 100 ml. volumetric flask, 1.0 gm. (10 mM) with 40 ml. of the 2 M hydroxylamine solution used in the method, wait 10 minutes, and fill to the mark with water. This stock solution may be kept for some time. 1 ml. of a 40 times

TABLE I

Relative Color Given by Various Compounds at Equal Concentration

Compound	Color
	per cent
Acetyl phosphate	100
Propionyl phosphate	98
Butyryl phosphate	97
Succinic anhydride	80
Acetamide	0.2
Glutamine	0 07
Ethyl acetate	0.04

diluted stock solution gives a color equivalent to 2.0 micromoles of acetyl phosphate. We found recrystallization unnecessary. The color was consistently 80 per cent of the acetyl phosphate equivalent.

Furthermore, it was found that a 0.005 M solution of acetamide, when heated in boiling water for 15 minutes with twice its volume of 5 M hydroxylamine hydrochloride in 4.5 N sodium hydroxide, yields quantitatively hydroxamic acid.[1] In this manner a standard solution of acethydroxamic acid may be prepared identical with standards obtained with acetyl phosphate. For this purpose, one takes 1.5 ml. of the heated solution and 1 ml. of acetate buffer plus 0.5 ml. of water and proceeds as described.

Interfering Substances—A number of anions depress the intensity of the color (Table II), presumably by forming iron complexes. We find that at

[1] Preliminary studies on a method for determination of acid amides gave encouraging results with acetamide. Unfortunately, however, the biologically interesting glutamine appears much less reactive with hydroxylamine.

least they do not interfere with the reaction between acyl phosphate and hydroxylamine. Thus fluoride in higher concentration causes appreciable depression and to a somewhat lesser degree phosphate, oxalate, and sulfate may interfere. Citrate was found without influence in similar concentrations. It seems possible, however, that other compounds, besides the ones tested, may have similar effects. In the cases considered, the permitted concentrations will generally be high enough to allow the use of the method without modification even in the presence of fluoride or phosphate. With higher concentrations of these anions, it was usually sufficient to use standard solutions containing analogous concentrations of the interfering anions. With very high concentrations, it is preferable to remove the fluoride and phosphate before the color is developed. For this purpose, we remove the protein with zinc hydroxide, adding calcium chloride to the zinc chloride solution. All volumes were doubled and after incubation with

TABLE II

Depression of Color Intensity by Certain Anions

Anion	Amount in 6 ml.	Depression
	micromoles	*per cent*
Fluoride	100	33
	50	7
	25	0
Phosphate	100	13
	50	4
Oxalate	150	14
	30	0
Sulfate	200	8

hydroxylamine 1 ml. was added of a solution containing 3.4 per cent in zinc chloride, 5.6 per cent in calcium chloride, and 1 ml. of 0.3 N sodium hydroxide. The combined protein, calcium, and zinc precipitate was removed. To 4 ml. of the filtrate 1 ml. each of the hydrochloric and ferric chloride solutions was added.

Examples for Determination of Biologically Formed Acetyl and Phosphoglyceryl Phosphate

A certain incompleteness was felt in the identification of metabolic acyl phosphates, since they had not been characterized by an organic chemical reaction typical for acid anhydrides. The instantaneous and quantitative conversion of acyl phosphates into hydroxamic acids furnishes now a test of the desired type. The present confirmation of the anhydride nature of metabolic acetyl and phosphoglyceryl phosphate thus completes the evidence already accumulated.

In Table III data are given for pyruvate oxidation with an extract of *Lactobacillus delbrueckii*. Oxygen consumption was measured manometrically and after 30 and 60 minutes, 0.15 ml. was taken to determine acetyl phosphate with the new method. The experiment is comparable to those reported earlier (5) with such enzyme preparations in which the phosphate precipitation method had been used. The initial phosphate concentration, however, was somewhat higher in the present case. Comparison between oxygen consumption and acetyl phosphate formation (last column, Table III) shows that the theoretical value of 1 (5) for the quotient (acetyl phosphate)/(oxygen) is reached here more closely than in earlier experiments. This close approach most probably is due to the presence of high phosphate concentration which inhibits acetyl phosphate breakdown (Utter and Werkman (6)) and furthermore to an avoidance in the present method of loss during deproteinization and subsequent manipulation.

TABLE III

Acetyl Phosphate Formation in Extract of Lactobacillus delbrueckii

1 ml. of bacterial extract, prepared as described previously (5), containing 0.15 M phosphate and 0.02 M fluoride, was shaken with air at 36° in the Warburg apparatus. 100 micromoles of pyruvate were added at zero time.

Incubation time	$-O_2$	Acetyl phosphate	$\frac{\text{Acetyl phosphate}}{O_2}$
min.	*micromoles*	*micromoles*	
30	11.2	10.6	0.95
60	20.1	19.1	0.95

Since the discovery of phosphoglyceryl phosphate by Warburg and his collaborators (7, 8), to our knowledge the only more extensive paper dealing with this substance is the report by Buecher (9) on the isolation of a yeast enzyme which catalyzes phosphate transfer between phosphoglyceric acid and adenyl pyrophosphate. In these studies a rather complex photometric method was used for the determination of phosphoglyceryl phosphate, based on its enzymatic reaction with reduced cozymase. With the hydroxylamine method phosphoglyceryl phosphate may be determined now with greater ease.

We were particularly eager to carry out experiments with muscle preparation, because the observations of Warburg's laboratory referred exclusively to yeast enzymes. Even in as crude a preparation as redissolved acetone precipitate of muscle extract, anhydride formation could be demonstrated easily with oxidation of phosphoglyceraldehyde as well as with the transfer of phosphate from adenyl pyrophosphate to phosphoglycerate. The acetone preparation used was prepared from cat muscle, according to Jandorf *et al.* (10). Data in Table IV are given in acetyl phosphate equivalents, since isolated phosphoglyceryl phosphate was not available for a determination of

the absorption coefficient. With crude yeast extracts similar results were obtained.

TABLE IV

Phosphoglyceryl Phosphate Formation in Muscle Extract

1 ml. samples of a 10 per cent solution of acetone precipitate of cat muscle extract were incubated in open test-tubes at 26°. The final volume was 2 ml. and all samples contained sodium fluoride in 0.02 M concentration. The substrates were added as the sodium salts; the given concentrations refer to final volume. The incubation time was 40 minutes.

Experiment No.	Substrates	Acyl phosphate
		micromoles
1	Hexose diphosphate, 0.04 M	0.6
	" " 0.04 " and pyruvate, 0.1 M	4.9
	Pyruvate, 0.1 M	0.3
2	Phosphoglycerate, 0.02 M	2.0*
	" 0.02 ", adenyl pyrophosphate, 0.02 M	10.7
	Adenyl pyrophosphate, 0.02 M	0

* Some creatine phosphate and adenyl pyrophosphate were present in the muscle extract.

TABLE V

*Acyl Phosphate Formation on Addition of Oxalacetate to Muscle Extract**

Conditions were the same as in the experiments of Table IV, except that fluoride was added as indicated below and the incubation time was 20 minutes.

Experiment No.	Additions	Acyl phosphate	Remarks
		micromoles	
1	None	0	0.02 M fluoride and 0.01 M adenyl pyrophosphate in all samples
	Pyruvate, 0.1 M	0.4	
	Oxalacetate, 0.03 M	3.6	
	Ketoglutarate, 0.05 M	0.5	
	Citrate, 0.02 M	0	
	Acetate, 0.04 M	0.3	
2	Oxalacetate, 0.03 M, fluoride, 0.02 M	2.6	
	Same + 0.02 M adenyl pyrophosphate	3.0	
	Oxalacetate, 0.03 M (no fluoride)	0.3	

* Oxalacetic acid was prepared from commercial ethyl oxalacetate by acid hydrolysis (11). According to the test with the aniline method at 26°, our preparation contained at least 90 per cent oxalacetic acid; titration corresponded to 100 per cent oxalacetic acid. Blanks of this preparation without enzyme showed no color; *cf.* also the experiment without fluoride on the last line of the table.

In order to explore anhydride formation with other acids, adenyl pyrophosphate was combined with a variety of metabolic intermediates. As shown in Table V, results were negative with the exception of oxalacetic

acid. From Experiment 2 in Table V, however, it appears that the color giving substance scarcely originates through phosphate transfer from adenyl pyrophosphate (*cf.* (12)); its formation occurs without addition of adenyl pyrophosphate and should rather be due to a metabolism of oxalacetate in the muscle preparation. Unlike acetyl phosphate, which is decomposed with great rapidity by muscle extract, the compound found with oxalacetate appears to be fairly stable and in this respect resembles phosphoglyceryl phosphate. It should be mentioned that besides acetyl and phosphoglyceryl phosphate, none of the known phosphate esters, including phosphopyruvate, gave a colored product with hydroxylamine, nor did oxalacetate without the muscle preparation. The fact that pyruvate does not react analogously seems to make it unlikely that the color with oxalacetate may be due to phosphoglyceryl phosphate, formed by oxidation of carbohydrate sources in the extract. As shown in Table V, fluoride is needed for formation or stabilization of the product.

SUMMARY

1. A micromethod is described for the determination of acyl phosphates. It is based on the conversion of these compounds into hydroxamic acids and colorimetric estimation of a purple ferric hydroxamic acid complex.

2. Experiments are reported, with this method, on acetyl phosphate formation with bacterial extracts, and phosphoglyceryl phosphate formation with crude muscle preparations. An unidentified substance yielding hydroxamic acid was found to appear when oxalacetate is added to muscle extract.

BIBLIOGRAPHY

1. Lipmann, F., and Tuttle, L. C., *J. Biol. Chem.*, **153**, 571 (1944).
2. Feigl, F., Anger, V., and Frehden, O., *Mikrochemie*, **15**, 18 (1934).
3. Degering, E. F., An outline of organic chemistry, 4th edition, New York, 327 (1943).
4. Summerson, W. H., *J. Biol. Chem.*, **130**, 149 (1939).
5. Lipmann, F., *J. Biol. Chem.*, **155**, 55 (1944).
6. Utter, M. F., and Werkman, C. H., *Arch. Biochem.*, **5**, 413 (1944).
7. Warburg, O., and Christian, W., *Biochem. Z.*, **303**, 40 (1939).
8. Negelein, E., and Brömel, H., *Biochem. Z.*, **303**, 132 (1939).
9. Buecher, T., *Naturwissenschaften*, **30**, 756 (1942); *Chem. Abstr.*, **38**, 5512 (1944).
10. Jandorf, B. J., Klemperer, F. W., and Hastings, A. B., *J. Biol. Chem.*, **138**, 311 (1941).
11. Krampitz, O. C., and Werkman, C. H., *Biochem. J.*, **35**, 595 (1941).
12. Lardy, H. A., Boyer, P. D., and Phillips, P. H., *Arch. Biochem.*, **6**, 295 (1944).

DENSITIES AND SIZES OF THE INFLUENZA VIRUSES A (PR8 STRAIN) AND B (LEE STRAIN) AND THE SWINE INFLUENZA VIRUS*

By D. G. SHARP, A. R. TAYLOR, I. W. McLEAN, Jr.,† DOROTHY BEARD, and J. W. BEARD‡

(From the Department of Surgery, Duke University School of Medicine, Durham, North Carolina)

(Received for publication, March 5, 1945)

Studies with the electron microscope have shown that the influenza viruses A (PR8 strain) (1) and B (Lee strain) (2) and the swine influenza virus (3) consist of rounded or bean-shaped particles of variable size and internal structure. The distribution of the sizes of the particles of a given type of the virus has been substantiated qualitatively by means of sedimentation velocity studies (4). Chemically the particles consist of protein, lipid, and carbohydrate with which there is associated nucleic acid of the desoxypentose type (5).

Estimates of the average size of the influenza virus particles have been made by means of the electron microscope and from sedimentation velocity data. In the initial work with the electron microscope, calibration was based on measurements of the widths of tobacco mosaic virus rods (1–3). Subsequent recalibration of the instrument has resulted in higher values (4) which are believed to be more nearly correct. Estimations of size were made (1–4) also from sedimentation velocity data in conjunction with values of the reciprocals of the partial specific volumes of the viruses determined with the pycnometer. It was recognized, however, that accurate knowledge of particle density in a medium compatible with the existence of the particle in its native state must be available if true sizes are to be calculated from sedimentation data.

* These investigations were aided through the Commission on Influenza, and the Commission on Epidemiological Survey, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Service, Office of the Surgeon General, United States Army. The work was aided also in part by a grant to Duke University from the Lederle Laboratories, Inc., Pearl River, New York.

† Member of the Commission on Epidemiological Survey, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Service, Office of the Surgeon General, United States Army.

‡ Consultant to the Secretary of War and a Member of the Commission on Acute Respiratory Diseases, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Service, Office of the Surgeon General, United States Army.

Probably the most direct method of determining the density of small particles in suspension is that of varying the density of the dispersion medium and noting the change in the sedimentation rate of the particles in a suitable centrifugal force field. Attempts have been made to use this method for studying the density of virus particles, materials such as sucrose, glycerol, urea, and sodium chloride being employed to vary the density of the suspending medium. It has been found (6–8) that under these conditions alterations occur in the behavior of the virus particles. In a recent report from this laboratory, however, a system was briefly described (9) in which influenza virus A (PR8 strain) was sedimented in bovine albumin solutions of various densities and the relationship obtained over the range of density available for study indicated an ideal behavior of the particles. In the present paper the method will be described in more detail, and the results obtained with influenza virus B (Lee strain) and the swine influenza virus will be given. These findings, together with data on the partial specific volume of these types of virus obtained in previous work and in the present study, will be discussed in connection with existing chemical and physical information to the end of obtaining a more accurate concept of the elementary influenza virus particle.

Materials and Methods

The three types of virus used were influenza viruses A (PR8 strain) and B (Lee strain) and the swine influenza virus, which were studied previously with respect to purification (1–3), chemical constitution (5), and physical properties (4).

Purified virus was obtained by concentration of the respective types of the virus from large pools of virus-infected chorioallantoic fluid from chick embryos by means of (1) adsorption on and elution from chicken red blood cells, followed by ultracentrifugation, or (2) preliminary concentration in the Sharples supercentrifuge, followed by ultracentrifugation.

Details of the procedures for preliminary concentration of the influenza viruses by adsorption on and elution from chicken red blood cells (1–3) and by large scale centrifugation in the Sharples centrifuge (10) have been reported. Further purification was then effected by treatment in the air-driven ultracentrifuge. Eluates or centrifugates in Ringer's solution (1) were spun in the angle centrifuge at 2000 *g*. The supernatants were then parceled into 15 ml. collodion tubes and spun for 1 hour at 20,000 *g* in the ultracentrifuge. The resulting clear, gel-like pellets were resuspended in Ringer's solution and spun at 5000 *g* in the ultracentrifuge for 5 minutes.

The concentrates exhibited a single, slightly diffuse boundary in the analytical ultracentrifuge (4) and a high degree of uniformity of particle kind in electron micrographs. In practically all instances such concentrates

of influenza virus A and the swine influenza virus gave values for the partial specific volume which did not change with further treatment of the virus. To obtain constant values with influenza virus B, however, further purifying ultracentrifugation was often necessary, particularly when preliminary concentration had been effected by centrifugation. The concentrates containing 100 to 400 mg. of virus were diluted to 120 ml. with Ringer's solution, parceled into 15 ml. ultracentrifuge tubes, and spun in the quantity ultracentrifuge head quickly up to and down from 6000 *g*. The virus was sedimented again at 20,000 *g* for 1 hour, resuspended at high concentration, and spun at 5000 *g* for 5 minutes.

The bovine albumin was a 25 per cent solution in 0.9 per cent NaCl solution of a crystalline fraction which Dr. Hans Neurath obtained from the Armour Laboratories, Chicago, Illinois, through the courtesy of Dr. E. J. Cohn, Harvard Medical School, and Dr. H. B. Vickery. To this albumin solution were added the calculated quantities of the salts requisite to make the solvent medium equivalent to Ringer's solution (11), consideration being taken of the volume actually occupied by the albumin itself. Dilutions of the stock albumin solution were made with Ringer's fluid. Virus in Ringer's solution at pH 7.3 to 7.5 was added to the bovine albumin of various concentrations, and the mixtures were brought to room temperature. To obtain virus in the highest concentration of albumin, 25 per cent, the appropriate amount of virus was sedimented in the ultracentrifuge and resuspended in the albumin solution. The concentration of virus in the albumin solution was 2.0 mg. per ml. of influenza virus A, 1.76 mg. per ml. of influenza virus B, and 3.0 mg. per ml. of the swine influenza virus. The time elapsing between addition of albumin and recording of the sedimentation diagrams was never greater than an hour and was generally about 20 minutes. The rotor temperature was recorded for each run.

Viscosity measurements on the albumin in Ringer's solution at 25° were made by Dr. John Erickson. It has been assumed that the change in viscosity of the albumin solution with temperature was the same as that for water. Any error introduced by this simplification would be small, because all centrifuge runs were made near 25° and the error would be random, for some runs were below and some above 25°.

Density of the albumin solutions was measured with the pycnometer in a thermostat at 25°.

For the studies with sucrose, a 64 per cent solution of sucrose (Pfanstiehl) in Ringer's solution was prepared. By appropriate mixture of this with Ringer's solution and concentrated virus, samples containing 3.0 mg. of virus per ml. with the required sucrose content were made immediately before the sedimentation run. Sucrose viscosity corrections were taken from the data of Bingham and Jackson (12), and the density values from

standard tables. Because of the presence of the salts of Ringer's solution, the viscosity and density values given in the tables are not precise in relation to the conditions of this experiment.

An air-driven ultracentrifuge (13) was used for the sedimentation velocity determinations. The mean rotor radius was 6.5 cm., and the cell height was 1.2 cm. The progress of sedimentation in the region of 25° was recorded by the Lamm scale method, and sedimentation rates were calculated as previously described (4).

For estimation of partial specific volume, the ultracentrifuge concentrates containing 14.0 to 23.0 mg. of virus per ml. were brought to room temperature (about 25°); air was removed by placing the vial containing the preparation in a small suction flask and evacuating three successive times to the point where the concentrate began to boil. A sample of the Ringer's solution was treated at the same time in order to control the slight concentration effect due to the evacuations. Virus concentration was then calculated by conversion of micro-Kjeldahl nitrogen determinations made in duplicate, with the appropriate factor for each type of virus (5). The calibrated 2 ml. capped pycnometer was then filled with chilled virus suspension and equilibrated in a water bath at 25° ± 0.05°. Weighings were made on a micro balance with calibrated weights, a duplicate pycnometer being used as a counterbalance. Solvent density determinations were taken from the average of at least three individual equilibrations and weighings. The partial specific volume was calculated by means of the equation given by Kraemer (14).

Results

The experiments have consisted of studies on (1) the sedimentation velocity of influenza viruses A (PR8 strain) and B (Lee strain) and swine influenza virus in bovine albumin solutions of different densities; (2) the sedimentation velocity of the swine influenza virus in sucrose solutions of different densities; and (3) the partial specific volumes of the three types of influenza virus estimated with the pycnometer. The studies with the pycnometer were made to obtain data to supplement those previously reported (4).

Sedimentation Velocity of Swine Influenza Virus in Sucrose Solutions—The results of the experiments with sucrose are given in Fig. 1, which shows the relation of the virus sedimentation rate, S, to the density, ρ_s, of the suspending medium. The ordinate, ηS, is the product of the observed sedimentation rate and the viscosity (relative to water at 25°) of the suspending medium. The abscissa is the density of the sucrose solution at 25°. The experimental points obviously are not distributed in a linear fashion, and through them the curve was drawn as described below. Extrapolation of

this curve would result in an intercept with the abscissa at about the value $\rho_s = 1.19$ for the density of the suspending medium.

From these findings, it is seen that the change in the sedimentation rate of the virus particles was not related wholly to the increase in the density of the medium. On the contrary, the curved nature of the relation of ηS to ρ_s shows that with increase in ρ_s, the values of ηS remained higher than would have been the case if the increase in the density of the suspending medium had been the sole influencing factor. It is thus evident that changes occurred in the physical properties of the virus particles which resulted in findings compatible with the assumption that particle density

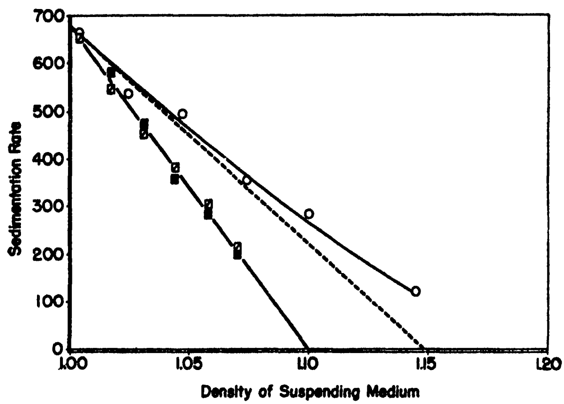


FIG. 1. Swine influenza virus. The sedimentation rates given in Svedberg units corrected for relative viscosity but not for density are plotted against the density of the suspending medium. Two different virus preparations were sedimented in bovine albumin solution, and the data are represented by the open and closed squares, respectively. One of these preparations (closed squares) was sedimented also in sucrose and the resulting data are shown by the open circles and the curve drawn through them. The tangent drawn to the curve at the density 0.9982 of the suspending medium is shown as a dotted line.

increased in relation to, though not in proportion to, the concentration of sucrose.

On further investigation of these changes in the sedimentation characters of the swine influenza virus, it was found that the extent of the alterations of the virus particles is dependent on the time of contact of the particles with the suspending medium. The results were qualitatively similar to those which were found with influenza virus A (PR8 strain), as illustrated in a previous report (9). Influenza virus A suspended in Ringer's solution containing 11 per cent sucrose exhibited at first a rapid and significant increase in sedimentation rate ((9) Fig. 1). With time, in this instance about 2.5 hours, the maximum value was obtained, and thereafter a gradual de-

crease in the sedimentation rate occurred. At the end of 28 hours, an interval not described in the previous report (9), the declining rate of sedimentation had reached a level close to that observed when the virus particles had been in contact with sucrose the shortest time possible for study.

Results qualitatively similar to these with the influenza viruses have been observed by Smadel, Pickels, and Shedlovsky (8) in studies on the elementary bodies of vaccinia. These authors, citing a number of possible explanations of the findings, concluded that the various phenomena were subject to elucidation on the assumption that the changes in particle behavior were due to osmotic withdrawal of water from the particles by the sucrose with consequent increase in particle density. It should be noted here for subsequent discussion that this hypothesis, while accounting for the increase in the sedimentation rate of the particles in a sucrose solution of given concentration, does not suffice to explain why there is a subsequent decrease with time.

The meaning of the curvilinear relations of Fig. 1 with respect to their bearing on the density of the virus particles in their native state is of uncertain significance. Interpretations have been made of such results by constructing a tangent to the curve (8) at the point corresponding to $\rho_s = 1.00$. The tangent is then extended to intercept the abscissa, and the value of the solvent density, ρ_s, at this intercept has been taken (8) to represent the density, ρ_v, of the virus particle in the absence of sucrose.

The observed data for the swine influenza virus shown in Fig. 1 are closely approximated by the relationship

$$\eta S = K_1 - K_2(\rho_s - 1) + K_3(\rho_s - 1)^2$$

which was used with appropriate constants to draw the curve. The slope of a tangent drawn at any point on the curve is then given by

$$\frac{d(\eta S)}{d(\rho_s - 1)} = -K_2 + 2K_3(\rho_s - 1)$$

which for small values of $(\rho_s - 1)$ should be nearly constant. The tangent drawn by this means through the point corresponding to the density of water at 20° ($\rho_s = 0.9982$) intercepts the axis at 1.148. Interpreted in the manner of Smadel, Pickels, and Shedlovsky, this result would indicate a density of 1.148 for the swine influenza virus in the absence of sucrose.

The significance of the tangent to the curvilinear relation is dependent on the validity of the assumption that the relation of ηS to $(\rho_s - 1)$ approaches a straight line in the region of $\rho_s = 1.00$ and, further, that such a linear relation would describe the behavior of the particles in a medium in which no change occurred in the character of the particles. In the investi-

gations with the swine influenza virus and, as well, with the elementary bodies of vaccinia (8), there were insufficient data either to show that the relation became linear in this region or for judging the *extent* of variation of the observed results from those expected under ideal conditions. Furthermore, a tangent drawn to the curve, ηS *versus* $(\rho_s - 1)$, may be used to calculate the density of the sedimenting unit at a particular value of ρ_s chosen only if ηS depends on ρ_s alone. On the contrary, however, it has been observed that ηS is not constant for a given ρ_s of the sucrose solutions but varies significantly with time. It is thus clear that measurements of ηS used to plot the curve ηS *versus* $(\rho_s - 1)$ will be different at different times and, consequently, such a calculated density of the sedimenting unit is likewise dependent on time. The sedimentation studies on the swine influenza virus, like those on the vaccinial elementary bodies (8), were made quickly after mixture of virus with sucrose solution, a period of time in which change in the virus particle is most rapid (8, 9). From these considerations and the results obtained with the bovine albumin experiments described below, then, it would appear unlikely that density values obtained from use of the tangent necessarily provide any information relative to the density of the particles in the native state.

Sedimentation of Influenza Viruses in Bovine Albumin Solution—Utilization of bovine albumin to vary the density of the suspending medium in the present experiments was prompted by the possibility that the change in the behavior of the virus particle in sucrose solutions may be due, as suggested, to the withdrawal of water from the virus particle caused by the osmotic pressure of the medium outside the particle. Addition of bovine albumin to Ringer's solution to the extent of 25 per cent increases the osmotic pressure of the solution by an amount approximately equivalent to the osmotic pressure of 0.002 M NaCl. In contrast, the osmotic pressure of a 25 per cent solution of sucrose corresponds approximately to that of 0.366 M (2.14 per cent) NaCl. The ratio of the osmotic pressure of bovine albumin to that of sucrose of the same percentage concentration is given by the inverse ratio of the respective molecular weights; namely, 342/70,000.

Experiments were done on the sedimentation of two different batches of swine influenza virus in a series of bovine albumin solutions of different densities. The results are shown in Fig. 1. The findings with two batches of influenza virus B are shown with similar symbols in Fig. 2. Data obtained with two batches of influenza virus A have been published elsewhere (9).

From inspection of these data as a whole, there is no consistent evidence of curvature of the relations between sedimentation rates, ηS, and the densities, ρ_s, of the suspending medium. Instead, the observed points appear to be arranged in an essentially linear fashion. For the group of data of

each type of the virus, a straight line was drawn through the points by the method of least squares. The various points are seen to lie close to the respective lines. The apparent goodness of fit is substantiated by the results of statistical analysis of the findings, which show that the deviation of the observed points from the straight line is well within the limits of error of

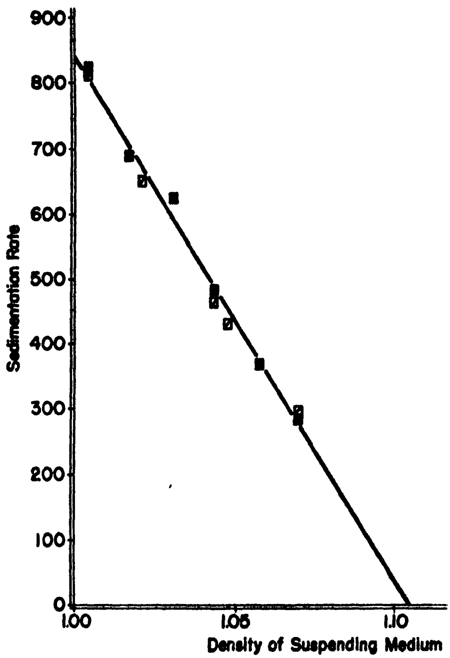


Fig. 2

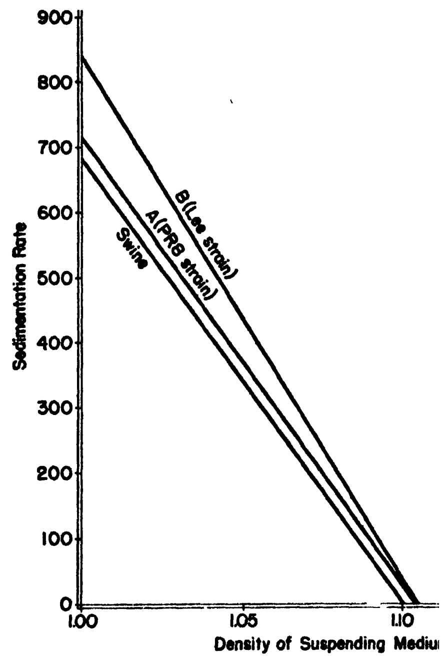


Fig. 3

Fig. 2. Influenza virus B (Lee strain). The sedimentation rates given in Svedberg units corrected for relative viscosity but not for density are plotted against the density of the albumin solution. Two different virus preparations were sedimented in bovine albumin solution and the data are represented by the open and closed squares.

Fig. 3. Summary of the sedimentation characteristics of the three types of influenza virus in bovine albumin solutions of different densities. The sedimentation rates, expressed in Svedberg units, were corrected for the relative viscosity but not for the density of the bovine albumin solution.

the method of centrifugal study. The lines obtained with the respective types of influenza virus are compared in Fig. 3.

In order to learn whether or not any change in ηS occurred with time of contact of the virus with the bovine albumin, the sample of influenza virus B (Lee strain) was studied at various intervals of time after mixing with the

highest concentration of bovine albumin used. The total elapsed times from mixing the virus with the 25 per cent albumin solution until sedimentation pictures were made were 48, 138, 226, and 313 minutes, for which the following corrected sedimentation rates, ηS, resulted: 309, 303, 323, and 313×10^{-13}, respectively. Although a possible increase was seen within the longer periods, it was thought likely that studies made within an hour would give values unaffected by possible changes due to the influence of the albumin. These are the same conclusions as those reached from data on influenza virus A (PR8 strain) at albumin density 1.037, as previously reported.

The degree of success of obtaining an estimate of the actual density of the virus particle in its native state by a method of this nature would be expected to be dependent on a number of factors. Electron micrographs show that the particles of the three types of influenza virus are approximately spherical. The sedimentation behavior of the particles would then be closely approximated by that of a sphere for which the equation of dynamic equilibrium for the centrifuge is as follows:

$$\frac{4}{3}\pi r^3(\rho_v - \rho_s)\omega^2 R = 6\pi\eta_1 r \frac{dR}{dt} \tag{1}$$

In this equation R is the distance at time t from a particle of radius r to the center of the centrifuge rotor. The angular velocity of the rotor is ω; the difference between the density of the virus and that of the suspending medium is $\rho_v - \rho_s$; and η_1 is the absolute viscosity of the suspending medium. Substitution of the sedimentation constant, which is by definition $S = (1/\omega^2 R)\,(dR/dt)$, in Equation 1 results in the simplification

$$\frac{2}{3}r^2(\rho_v - \rho_s) = 3\eta_1 S \tag{2}$$

From Equation 2 it is seen that under ideal conditions a linear relationship should exist between density of the suspending medium and the product $\eta_1 S$ for any given particle density, ρ_v, and that $\eta_1 S \rightarrow 0$ as $(\rho_v - \rho_s) \rightarrow 0$, indicating the limiting density of the virus particle.

The applicability of the theoretical considerations to estimation of the density of the virus particles is dependent chiefly on the constancy of both the size and the density of the particles despite the changes in the density of the dispersing medium. As noted above, the influenza virus particles are close enough to the spherical shape to fulfil the conditions of Stokes' law. Direct evidence of whether the size and density of the virus particles did or did not change is not available. However, the relation of the corrected sedimentation rate to the density of the albumin solution is essentially linear. If the relation is actually linear, it may be concluded, as

indicated in Equation 1, that no change occurred in the size of the particle. A possible change in particle density is highly improbable, since such a change would have had to be a linear function of the density of the dispersing medium. Further evidence for the absence of change within the conditions of the experiments was obtained in studies on both influenza virus A (PR8 strain) as previously reported and on the influenza virus B (Lee strain) in the present work.

Assuming that the relation is linear, for which the data provide good evidence, calculations were made of the particle density of the three types of influenza virus. For this Equation 2 was used, and the resulting values are shown in Table I. These values, 1.104, 1.104, and 1.100, represent the den-

TABLE I

Sedimentation Constant, Density, Mean Diameter, Partial Specific Volume, and Water Content of Influenza Viruses

	Influenza virus A (PR8 strain)	Influenza virus B (Lee strain)	Swine influenza virus
Sedimentation constant*	742×10^{-13}	840×10^{-13}	727×10^{-13}
Density in aqueous suspension	1.104	1.104	1.100
Diameter, from sedimentation velocity data, mμ	116	124	117
Diameter, from electron micrographs; direct calibration, mμ*	101	123	96.5
Partial specific volume	0.822	0.863	0.850
Water content, *% by volume*	52.0	34.5	43.3

* Data previously published (4).

sities of the sedimenting units of influenza viruses A and B and the swine influenza virus, respectively, including water moving with the particles. The sizes of the particles under these conditions may be calculated from their densities as given in Table I and the respective sedimentation constants obtained by extrapolation to infinite virus dilution as previously reported (4). The average sizes obtained in this way (Table I) were 116, 124, and 117 mμ, respectively. A close approximation to the particle sizes may be obtained directly from the data on sedimentation in albumin solution from the slopes of the lines of Fig. 3. The slopes of the respective lines are given by the first derivative of Equation 2

$$\frac{d(\eta_1 S)}{d\rho_s} = -\frac{2}{9}r^2 \tag{3}$$

from which it is seen that

$$3\sqrt{\frac{-\text{slope}}{2}} \tag{4}$$

Although Equation 4[1] is attractively simple, it gives values in these experiments which are 3 to 5 per cent lower than those calculated as described above by using values of S obtained from a separate experiment extrapolated to zero concentration of virus and the densities calculated from the straight lines of Fig. 3. The reason for this probably lies in the dependence of S on virus concentration, which tends to reduce the slope of the line. This should, however, have no influence on the determination of limiting density, for its influence diminishes with S and approaches zero with it.

Partial Specific Volumes of Influenza Viruses—The values obtained for the partial specific volumes of the three types of virus are given in Table II. Each tabulated value is the average of two or three independent replicate

TABLE II

Partial Specific Volumes of Influenza Viruses

Influenza virus A (PR8 strain)				Influenza virus B (Lee strain)				Swine influenza virus			
Preparation No.	Ultracentrifugal cycle	Virus concentration	Partial specific volume	Preparation No.	Ultracentrifugal cycle	Virus concentration	Partial specific volume	Preparation No.	Ultracentrifugal cycle	Virus concentration	Partial specific volume
*		*mg. per ml.*				*mg. per ml.*				*mg. per ml.*	
21	1	10.85	0.823	30	1	14.43	0.814	26	1	16.85	0.850
21	2	5.60	0.822	30	2	13.00	0.862	26	1	12.25	0.852
23	1	22.3	0.820	30	3	13.30	0.861	26	1	7.98	0.850
23	2	20.3	0.822	30	3	13.30	0.862				
				30	3	9.07	0.862				
				32	1	18.06	0.800				
				32	2	16.66	0.840				
				32	3	7.72	0.863				
				30-32*	4	9.05	0.862				

* Preparations 30 and 32 were combined for the fourth ultracentrifugal cycle as explained in the text.

determinations at the indicated virus concentration and ultracentrifugal cycle. The results were essentially identical with those previously reported (4). With the influenza virus A no difficulty was encountered in obtaining ultracentrifugal concentrates from either red blood cell eluates or centrifugates which gave constant values. The same was generally true with the virus of swine influenza. In the instance of influenza virus B, however, the concentrates required at least two and often three cycles of ultracentrifugation before a constant value for the partial specific volume was reached.

[1] The negative sign under the radical will disappear when the observed negative slope is introduced in the equation. Relative viscosity (η) must be changed to the absolute value (η_1) for this calculation.

Preparation 30 gave a value of 0.862 after the second ultracentrifugal sedimentation, and this was not changed by additional sedimentation. Preparation 32 gave the value 0.863 only after three ultracentrifugal sedimentations. Since there was insufficient material remaining of either Preparation 30 or 32 to permit further ultracentrifugation, the two preparations, both having had three ultracentrifugal cycles and both giving essentially identical values for partial specific volume, were combined for a fourth ultracentrifugal cycle. As shown in Table II, no significant change in specific volume was observed following the fourth cycle.

Water Associated with Virus Particles—By combining the results of the measurements on partial specific volume with the density of the virus particles determined from the studies with bovine albumin, the amounts of water associated with the virus particles were calculated. The values, Table I, were for influenza virus A (PR8 strain) 52 per cent, influenza virus B (Lee strain) 34.5 per cent, and the swine virus 43.3 per cent by volume. The value, 66 per cent, previously reported (9) for influenza virus A (PR8 strain), was calculated from essentially the same data that are presented here. The difference was due to an error in the method of calculation. The present value for this virus, 52 per cent, is likewise somewhat lower than the value, 60 per cent, found by Lauffer and Stanley (15).

DISCUSSION

The behavior of the influenza viruses centrifuged in bovine albumin solutions revealed no consistent evidence of change in the sedimentation characters of the particles within the conditions of the experiments. This is in marked contrast to the pronounced changes occurring in the presence of sucrose, which have been interpreted (6–8) to be due to an increase in density and a possible decrease in particle size as the result of the osmotic influence of the suspending medium. The results of the experiments with bovine albumin solutions of high density but of low osmotic pressure provide strong presumptive evidence of the verity of the hypothesis. Within the limits that the data indicate an ideal behavior of the particles, it is possible, without further assumption, to employ the findings directly for the calculation of the density of the particles in their native state.

Use of bovine albumin for virus density determinations presents certain limitations of both theoretical and practical nature. It is possible that the relation of sedimentation rate to the density of the albumin solutions is not wholly linear. It is conceivable that the virus particles become coated with albumin and thus increase in size and density. Further, the density of the dispersing medium feasible for study is somewhat limited, since bovine albumin solutions of densities greater than 1.07 are of high viscosity. The high centrifugal fields necessary to sediment the virus through the denser

albumin solutions tend to cause sedimentation of the albumin. For this reason it is possible that the lower virus sedimentation values (ρ_s near 1.07) may be somewhat higher than their true level, yielding densities of the virus particles slightly greater than the true values.

The densities observed were 1.104, 1.104, and 1.100 for the influenza viruses A and B and the swine influenza virus, respectively. These values obtained in the studies with bovine albumin were far lower than the value suggested by the results with sucrose solutions for the swine influenza virus. The tangent drawn mathematically to the curve of the experimental data, Fig. 1, intercepted the abscissa at the point corresponding to the density 1.148 of the suspending medium. The experimental data observed for influenza virus A with sucrose by Lauffer and Stanley (15) were similar to those found for the swine virus with sucrose in the present work. The curve of the latter, on extrapolation, intercepted the abscissa at solvent density 1.19, whereas that described by Lauffer and Stanley intercepted at 1.18. In interpreting their results, however, these authors stated: "A tangent to the experimental curve at the point corresponding to the density of water, drawn in the manner outlined by Smadel, Pickels, and Shedlovsky, will describe the sedimentation behavior which the particles would exhibit if they did not change density with increasing sucrose concentration. Such particles would just float in a solvent of density equal to about 1.1, hence it can be inferred that the density of PR8 influenza virus particles in the absence of sucrose is about 1.1." On the basis of their results, which are shown by the points and the curve reproduced in part in Fig. 4 from an enlarged photograph of their Fig. 3 (15), it is not clear how such an inference could be drawn. The findings of these authors have been analyzed by deriving the equation of the curve from the observed points as was done in the instance of the swine influenza virus. From the value of the first derivative of the equation at the point of the density of water at 20°, 0.9982, the tangent was drawn as shown in the broken line of Fig. 4. The value of the intercept with the abscissa, 1.143, is greatly different from that "inferred" by Lauffer and Stanley, but, incidentally, is nearly identical with the value obtained in the present work with sucrose for the swine influenza virus.

The values of the partial specific volumes observed are of an order of magnitude in accord with the composition of the dehydrated virus particles (5). The selection of an accurate method for the determination of the partial specific volume of chemical complexes such as the influenza viruses warrants careful consideration. The removal of salts from the suspending medium by dialysis, as carried out by Lauffer and Stanley (15), results in precipitation of the influenza viruses. Owing to the difficulty of maintaining uniform dispersion, such precipitated material is not suitable for accurate pycnometric determinations. The classical toluene displacement

method is likewise inapplicable, since the virus contains lipid as an integral part of the complex. For these reasons the present studies were made on concentrated virus preparations in saline medium (Ringer's solution). With virus concentrates containing 8 to 25 mg. of virus per ml., the differences between the weights of the suspending medium and those of the virus concentrates were of the order of 1000 to 8000 γ on the micro balance, and the replicate weights of a given preparation were reproducible to within 10 γ. The greatest source of error is in the Kjeldahl nitrogen determination and the conversion of nitrogen to virus concentration. Constant boiling HCl and calibrated pipettes and burettes were used, and the duplicate nitrogen determinations showed better than 1 per cent agreement. This

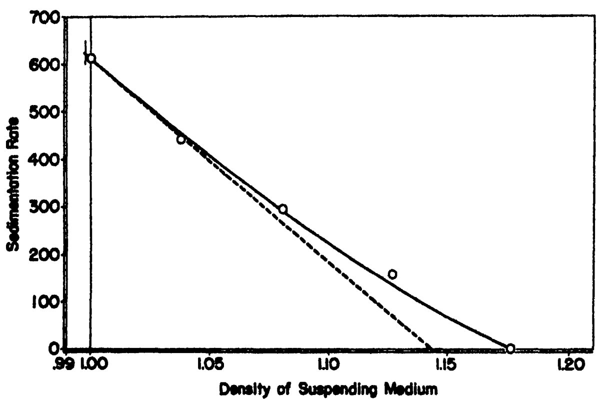


FIG. 4. Influenza virus A (PR8 strain). The sedimentation rates are given in Svedberg units with suitable correction for the viscosity of the suspending medium. The sedimentation rates are plotted against the density of the sucrose suspending medium. The data were taken from those of Lauffer and Stanley (15). The tangent at $\rho_s = 0.9982$, which was not given in the original publication, has been constructed by the present authors as shown in the dotted line.

Kjeldahl nitrogen conversion error should be relatively negligible at the high virus concentrations used.

The specific volume of any pure substance should be constant if the complex is an independent chemical and physical entity of constant constitution. The present results indicate that the influenza virus particles are entities of this nature. The composition of a given type of influenza virus is uniform (5) with respect to the proportions of protein, lipid, and carbohydrate, though these proportions, particularly of the lipid, vary to a slight but probably significant degree with the type of the virus. The values of the partial specific volumes likewise reveal distinct type differences. Moreover, a definite limiting value characteristic for each type was reached which did not change with repeated ultracentrifugation nor with dilution.

Lauffer and Stanley (15) regarded a "value of 0.79 as the most probable value for the true specific volume of PR8 influenza virus preparations obtained by differential centrifugation." Only one of their preparations was examined directly in electrolyte solution, and the value observed, 0.80, approaches the value seen with influenza virus A in the present work. Two other preparations examined by these authors gave values of 0.84 and 0.85, which are higher than any obtained in this laboratory with this type of the virus.

From the present findings certain implications may be drawn relative to the nature of the influenza virus particles. Chemically they are constituted of protein, fat, carbohydrate, and, presumably, inorganic salts with which water is associated. The nature of the association of water with the particles is such that the water is drawn rapidly from the particles in the presence of sucrose in solution. This result is explained simply by the assumption that the withdrawal of water is due to the osmotic influence of the sucrose. Substantiation of this possibility is seen in the finding that comparable changes do not take place in bovine albumin solutions of low osmotic pressure. As noted in a section above, however, this assumption does not explain all of the findings, for it was observed that after a period of increase in the apparent density of the virus particles in sucrose solution there followed a definite and substantial decrease in apparent density. This phenomenon was noted also by Smadel, Pickels, and Shedlovsky (8) in studies on the elementary bodies of vaccinia. In order to explain the findings as a whole, it would appear necessary to postulate a membrane or membrane-like structure enveloping the particle which is permeable to sucrose but impermeable to bovine albumin. Under these conditions the particles placed in sucrose would first lose water and thus increase in density. At the same time sucrose would begin to enter the particle, and after an initial period of water loss the concentration of sucrose inside the particle would reach a level sufficient to reverse the flow of water, which would result in subsequent decrease in density. These possibilities are closely in accord with the actual experimental findings in the present work and with results previously reported (9).

It seems plain that the influenza viruses are not molecular in nature. Instead, there is mounting evidence that they exhibit a behavior and a structure which strongly suggest that these viruses are relatively highly organized cell-like bodies limited by a definite membrane-like structure similar in certain of its properties to the analogous semipermeable cell wall of bacteria.

The authors are greatly indebted to Mr. Aaron Herr for his aid in the analysis of the Lamm scale diagrams.

SUMMARY

The densities of influenza viruses A (PR8 strain) and B (Lee strain) and the swine influenza virus were estimated from the results of sedimentation in bovine albumin to be 1.104, 1.104, and 1.100, respectively. Because of the apparently ideal behavior of the virus particles under the conditions of the experiments, these values are considered to represent the densities of the particles in their native state. The average sizes of the particles, calculated from these data, were 116, 124, and 117 mμ, respectively. The true partial specific volumes determined by pycnometer measurement were 0.822, 0.863, and 0.850. From the density values and the partial specific volumes, the amounts of water associated with the respective types of the virus were 52, 34.5, and 43.3 per cent by volume.

Studies were made also on the sedimentation velocity of the swine influenza virus in sucrose solutions. The results obtained, together with findings with influenza virus A previously reported, are discussed in relation to the results of the studies with bovine albumin solution.

BIBLIOGRAPHY

1. Taylor, A. R., Sharp, D. G., Beard, D., Beard, J. W., Dingle, J. H., and Feller, A. E., *J. Immunol.*, **47**, 261 (1943).
2. Sharp, D. G., Taylor, A. R., McLean, I. W., Jr., Beard, D., Beard, J. W., Feller, A. E., and Dingle, J. H., *J. Immunol.*, **48**, 129 (1944).
3. Taylor, A. R., Sharp, D. G., McLean, I. W., Jr., Beard, D., Beard, J. W., Dingle, J. H., and Feller, A. E., *J. Immunol.*, **48**, 361 (1944).
4. Sharp, D. G., Taylor, A. R., McLean, I. W., Jr., Beard, D., and Beard, J. W., *J. Biol. Chem.*, **156**, 585 (1944).
5. Taylor, A. R., *J. Biol. Chem.*, **153**, 675 (1944).
6. Bechhold, H., and Schlesinger, M., *Biochem. Z.*, **236**, 387 (1931).
7. Schlesinger, M., *Z. Hyg. u. Infektionskr.*, **114**, 161 (1932–33).
8. Smadel, J. E., Pickels, E. G., and Shedlovsky, T., *J. Exp. Med.*, **68**, 607 (1938).
9. Sharp, D. G., Taylor, A. R., McLean, I. W., Jr., Beard, D., and Beard, J. W., *Science*, **100**, 151 (1944).
10. Taylor, A. R., Sharp, D. G., McLean, I. W., Jr., Beard, D., and Beard, J. W., *J. Immunol.*, in press.
11. Bayliss, W. M., Principles of general physiology, London and New York, 4th edition (1927).
12. Bingham, E. C., and Jackson, R. F., *Bull. Bur. Standards*, **14**, 59 (1918–19).
13. Wyckoff, R. W. G., and Lagsdin, J. B., *Rev. Scient. Instruments*, **8**, 74 (1937).
14. Kraemer, E. O., in Svedberg, T., and Pedersen, K. O., The ultracentrifuge, Oxford, 325 (1940).
15. Lauffer, M. A., and Stanley, W. M., *J. Exp. Med.*, **80**, 531 (1944).

AN ESTIMATION OF ACETIC ACID FORMATION IN THE RAT*

By KONRAD BLOCH AND D. RITTENBERG

(*From the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York*)

(Received for publication, March 12, 1945)

The finding that the feeding to animals of deuterio acetate leads to the formation of deuterio cholesterol (1) not only sheds light on the nature of sterol precursors but also demonstrates that either acetic acid itself or a derivative into which it can readily be converted occurs normally in intermediary metabolism. Since the extent to which acetate is formed during the intermediary metabolism of the major dietary constituents was unknown, the only quantitative conclusion as to the acetate-sterol transformation was that, under our experimental conditions, a minimum of 6 per cent of all hydrogen atoms in cholesterol had been derived from acetate. This value was reached without allowance for the probable dilution of the labeled dietary acetate by acetic acid formed in intermediary metabolism or for possible loss of deuterium by an exchange reaction.

Although it has been shown that acetic acid is formed in tissue slices by dismutation of pyruvate (2), direct proof for its formation as a normal metabolite in the intact animal has not yet been furnished. Since acetic acid is metabolized at a rapid rate, as has been demonstrated by feeding experiments with acetic acid containing heavy carbon (3), its concentration in animal tissues at any instant may be too small to permit detection though the amount produced per day may be large.

Four biochemical conversions involving acetic acid have been demonstrated; *viz.*, condensation to acetoacetate (4, 5), formation of cholesterol (1), of fatty acids (6), and acetylation of foreign amino acids or amines (7, 8).[1] When isotopic acetate is fed, the products contain considerably lower isotope concentrations. This dilution may be due to a number of causes. The isotopic acetate, before entering into intermediary reactions, merges with endogenous non-isotopic acetate. Secondly, if precursors other than acetate exist, the hydrogen atoms of the product derived from those sources will lower the isotope concentration. Thirdly, deuterium may be lost as a result of exchange reactions. Further, in the case of a

* This work was carried out with the aid of grants from the Josiah Macy, Jr., Foundation and from the Nutrition Foundation, Inc.

[1] From the red blood cells of rats fed deuterio acetate we have isolated hemin and have found it to contain significant concentrations of deuterium. This finding is of interest in connection with the fact that condensations involving acetoacetic ester and ammonia yield pyrroles (Knorr synthesis (9)).

substance, like cholesterol, which is synthesized slowly (10), the newly formed compound will be diluted with the non-isotopic compound already present.

If in a synthetic reaction acetic acid is the sole precursor, the final isotope concentration of the product should be a measure of the dilution of the dietary acetic acid by that formed in the animal organism at the site of the acetylation. Bernhard has shown, with the aid of deuterio acetate, that acetic acid can be directly utilized in the formation of N-acetyl groups of foreign amino acids and amines (7). The dilution of isotope observed in this process may, therefore, give at least an indication of the minimum extent of endogenous production of acetic acid by the rat. In the present investigation of the acetylation reaction the dilutions proved to be inversely proportional to the level of dietary acetate but were independent of the amount of amine administered. Similar values were obtained when either *dl*-γ-phenyl-α-aminobutyric acid, one of its optical antipodes, sulfanilamide, or *p*-aminobenzoic acid was employed as the test substance for acetylation.

Our data definitely show that the acetylation reaction does not involve loss of carbon-bound deuterium. We have fed a preparation of isotopic acetate which was labeled both by carbon-bound deuterium in the methyl group and by heavy carbon (C^{13}) in the carboxyl group. The dilutions of deuterium and of C^{13} in the acetyl group of the excreted acylamino acid were identical, for the ratio of the two isotopes remained constant. Although there is no doubt that acetic acid is an effective acetylating agent, the possibility that acetyl groups are also derived from other sources had to be explored. In order to test whether, as suggested originally by Knoop (11), a mechanism involving condensation of pyruvic acid with the amine could give rise to acetyl groups, we have administered deuterio alanine as a source of pyruvic acid. When *l*- or *dl*-phenylaminobutyric acid was the foreign amine, deuterio acetyl derivatives with high isotope concentrations were excreted; on the other hand, when *p*-aminobenzoic acid or sulfanilamide was administered the acetyl groups contained very low concentrations of deuterium. Pyruvic acid can therefore act as a direct source of acetyl groups for the foreign α-amino acid but not for the aromatic amines.

The results here presented furnish data on the quantitative rôle of acetic acid as an acetylating agent and illustrate the application of the isotope dilution technique to the measurement of the production of acetic acid in animal tissues.

EXPERIMENTAL

Preparation of Deuterio Acetic Acid—Deuterio malonic acid was prepared by exchange with heavy water and decarboxylated to deuterio acetic acid (12). The sodium salt was fed.

Preparation of Deuterio C^{13}·Acetic Acid, CH_3—COOH—CO_2 containing 2.34 atom per cent excess C^{13} was treated with methyl magnesium iodide. The acetic acid obtained by the usual methods was converted to silver acetate. Ag, calculated, 64.7 per cent; found, 63.4 per cent. It contained 1.13 atom per cent excess C^{13}.

The silver acetate was converted into the sodium salt and deuterium was introduced into the latter by heating with heavy water (99.5 atom per cent excess D_2) in a sealed flask at 125°, in the presence of active platinum, for a period of 5 days. The sodium acetate contained 42.5 atom per cent deuterium. Ratio, $D:C^{13} = 42.5:1.13 = 37.6$.

Preparation of Deuterio dl-Alanine—Deuterio alanine was prepared by reduction of pyruvic acid with D_2 gas and palladium black in ethanolic solution of ammonia (13) (Preparation I). Another sample was synthesized by bromination of α, β-dideuterio propionic acid and reaction of the bromo derivative with ammonia (Preparation II). Preparation III was prepared from deuterio acetaldehyde and ammonium cyanide. In order to determine the isotope concentrations at the β-carbon atom, samples of the three alanine preparations were oxidized to acetaldehyde by chloramine-T and the acetaldehyde to acetic acid by $KMnO_4$ (8). The hydrogen atoms at the β-carbon atom of the three different alanine samples contained as follows: Preparation I 22.0, Preparation II 30.0, Preparation III 60.7 atom per cent excess deuterium.

dl-γ-Phenyl-α-aminobutyric Acid—The compound was prepared according to du Vigneaud and Irish (14). Nitrogen (Kjeldahl), 7.8; calculated, 7.8.

l(+)-Phenylaminobutyric Acid—The $l(+)$-γ-phenyl-α-acetaminobutyric acid excreted by rats which had been given the racemic amino acid was hydrolyzed by heating with 2 N HCl. The *l*-phenylaminobutyric acid isolated had a rotation of $[\alpha]_D = +46.0°$; 2 per cent in N HCl. N (Kjeldahl) found, 7.7 per cent; calculated, 7.8.

d(−)-Phenylaminobutyric Acid—In the resolution of the racemic phenylaminobutyric acid the procedure of du Vigneaud *et al.* was followed (15), the isomeric phenylethylamine salts of the carbobenzoxy derivative of the amino acid being used. The *d* isomer obtained had a rotation of $\alpha_D = -45.5°$, for a 1 per cent solution in N HCl. N (Kjeldahl) found, 7.8; calculated, 7.8.

Animal Experiments—In all experiments adult male white rats weighing between 200 and 300 gm. were used. The animals were kept in metabolism cages and fed a stock diet containing 71 per cent starch, 15 per cent casein, 3 per cent cottonseed oil (Wesson oil), 2 per cent cod liver oil, 5 per cent yeast, 4 per cent salt mixture (16). The animals received 12 to 15 gm. (according to their weights) of this stock diet to which was added the sodium salt of isotopic acetate, phenylaminobutyric acid,

p-aminobenzenesulfonamide, or p-aminobenzoic acid. Urine was collected until 24 hours after the last administration of food, and pooled. At the end of the experimental period the funnels for urine collection were washed with 1 per cent sodium hydroxide and the washings combined with the pooled urine.

Isolation of l-Acetylphenylaminobutyric Acid—The isolation of the acetyl derivative was carried out as previously described (8). The product was recrystallized until it melted at 179–180° (corrected). N calculated, 6.33; N found for all samples, 6.2 to 6.4. The yields of pure acetyl derivative recovered from the urine were 30 to 35 per cent after the feeding of *dl*-phenylaminobutyric acid, 40 to 45 per cent after the feeding of $l(+)$-phenylaminobutyric acid, and 20 to 25 per cent after the feeding of $d(-)$-phenylaminobutyric acid (15). The rotation was determined in three typical experiments: (*a*) acetyl derivative isolated after feeding *dl*-phenylaminobutyric acid (Experiment 3, Table I), $[\alpha]_D = +27.7°$ (2 per cent in ethanol); (*b*) acetyl derivative isolated after feeding $l(+)$-phenylaminobutyric acid (Experiment 3, Table II), $[\alpha]_D = +27.5°$ (2 per cent in ethanol); (*c*) acetyl derivative isolated after feeding $d(-)$-phenylaminobutyric acid (Experiment 4, Table II), $[\alpha]_D = +28.3°$ (2 per cent in ethanol).

Isolation of Acetyl-p-aminobenzoic Acid—The samples of urine were made alkaline to phenolphthalein and extracted continuously with ether for 5 hours. They were then acidified to Congo red and extracted continuously with ether for 24 hours. The ether extract from the acidified urine was concentrated and the residue dissolved in a small volume of N H_2SO_4, when acetyl-p-aminobenzoic acid crystallized. The product was recrystallized from water with addition of charcoal until the melting point was 255–260° (uncorrected), with decomposition. N calculated, 7.82 per cent; found for all samples, 7.7 to 7.9 per cent.

Isolation of Acetylsulfanilamide—The procedure of Bernhard (7) was followed. The samples were recrystallized from water until they had a melting point of 217–218° (corrected). N calculated, 13.1 per cent; found, 13.1 to 13.3 per cent.

The quantities of acetylsulfanilamide or acetyl-p-aminobenzoic acid which could be isolated in pure form varied in different experiments, and in most cases were less than 5 per cent of the amount of amine fed.

Isolation of Cholesterol—Cholesterol was isolated either as the digitonide or as free cholesterol, as described before (1).

The deuterium concentrations of the acetyl groups were obtained (Tables I to III) by determining the deuterium concentrations of the acetyl compounds and assuming that all the deuterium was present in the acetyl group. No attempt was made to analyze acetic acid after hydrolysis

of the acetyl derivative, since acetic acid exchanges slowly at elevated temperatures (17). It was shown, however, that the isotope in the acetylamino acid was present almost entirely in the acetyl group. A sample of isotopic acetylphenylaminobutyric acid isolated from one of the feeding experiments was hydrolyzed by refluxing with 2 N HCl and the free phenylaminobutyric acid analyzed. It contained no excess of deuterium.

The observation by Fishman and Cohn (18) that the acetyl group of the excreted acetylphenylaminobutyric acid contains deuterium when the body fluids of the animals are enriched with D_2O made it seem possible that some of the isotope content of the acetyl groups in the present experiments could have been due to the introduction of deuterium from the body fluids. However, the deuterium concentrations of the body fluids in the present experiments were always quite low. No appreciable error is therefore introduced by disregarding the deuterium content of the amine. The possibility that some of the isotope in the excreted acetyl derivative might have been lost by exchange during the isolation procedure was eliminated by the following experiment. A solution of 0.500 gm. of normal *dl*-acetylphenylaminobutyric acid in 50 ml. of D_2O (3 atom per cent excess D) was refluxed for 5 hours. When cold, the acetyl derivative was filtered, washed with ordinary water, and reprecipitated after solution in dilute alkali. It contained no excess deuterium.

Analysis of Isotopic Acetylphenylaminobutyric Acid Containing C^{13}—0.25 gm. of *l*-acetylphenylaminobutyric acid isolated after the feeding of sodium acetate containing 42.5 atom per cent excess deuterium and 1.13 atom per cent excess C^{13} was hydrolyzed by refluxing in 2 N sulfuric acid for 3 hours. Acetic acid was distilled from the reaction mixture and converted into silver acetate. Ag content, found, 63.7; calculated, 64.7.

For C^{13} analysis the silver acetate was burned and the CO_2, analyzed in the mass spectrograph, showed 0.057, 0.060 atom per cent excess C^{13}. A second sample of acetylphenylaminobutyric acid isolated from the same experiment was analyzed for deuterium. The acetyl group contained 2.28 atom per cent excess deuterium. The ratio of D to C^{13} in the acetyl group was therefore 2.28:0.058 = 39.3; in the sodium acetate administered the ratio was 42.5:1.13 = 37.6.

DISCUSSION

When deuterio acetate is administered the excreted acetylamino compound has a lower deuterium concentration than the dietary acetate. The stability *in vivo* of the carbon-hydrogen bond in acetic acid was proved by the experiment in which the dietary acetate was labeled with both deuterium and C^{13}; the ratio of the isotopes in the acetyl groups of the excreted amino acid (39.3) was within experimental error, the same as in the acetate

fed (37.6). The stability *in vitro* of the carbon-hydrogen bond of acetic acid at room temperature (17) thus persists *in vivo*.

In Table I are listed the deuterium concentrations of the acetyl groups of the acetylphenylaminobutyric acid excreted after administration of deuterio acetate. The data show that the isotope dilution factor, *i.e.* the ratio of the percentage of D in the acetate fed to that in the acetyl group, is inversely proportional to the quantity of acetate fed per unit of body weight. If the dosage in one experiment is twice as high as in another, the isotope concentrations of the excreted acetyl groups will also be twice as high. The product of the dosage and the dilution factor is a constant.[2]

TABLE I

Deuterium Concentrations in Acetyl Groups of Acylamino Acids Excreted after Simultaneous Feeding to Rats of Sodium Deuterio Acetate and dl-γ-Phenyl-α-aminobutyric Acid

Experiment No.	Deuterio acetate fed per day (a)	Deuterio acetate fed per day (b)	*dl*-Phenyl-amino-butyric acid fed (c)	Duration of feeding	Deuterium concentration in Body water	Deuterium concentration in Acetyl group (d)	Dilution factor $\frac{(b)}{(d)}$	Product, dosage × dilution factor $\frac{(a) \times (b)}{(d)}$
	mM per 100 gm. body weight	*atom per cent excess deuterium*	*mM per 100 gm. body weight*	*days*	*atom per cent excess*	*atom per cent excess*		
1	0.078	68.0	0.45	5	0.02	0.22	309	24.1
	0.078	68.0	0.45	5	0.02	0.24	283	22.1
2	0.81	54.1	0.10	10	0.07	1.88	28.8	23.3
3	1.22	42.5	0.45	5	0.06	2.30	18.5	22.5
4	1.52	27.6	0.55	4	0.07	2.10	13.1	19.9
	1.52	27.6	0.55	6	0.07	1.92	14.4	21.9
	1.52	27.6	0.55	8	0.07	1.95	14.1	21.4
5	1.60	54.1	0.55	4		4.57	11.8	18.9
	1.60	54.1	0.55	4		4.58	11.8	18.9
6	1.68	6.9	0.44	5	0.02	0.60	11.5	19.3
	1.68	6.9	0.44	5		0.63	11.0	18.5

This relation holds over a wide range. In our present experiments a 20-fold variation of the level at which acetate is administered did not affect the constancy of this product.

In Experiment 4, Table I, three rats received the same daily addition of acetate, one of the animals for 4, another for 6, and the third for 8 days. The isotope concentrations in the acetyl groups excreted in all three cases were the same. This indicates that storage of acetate or of the acetylating

[2] From theoretical considerations we should expect the value, (dilution factor minus 1) × amount of acetate fed per unit of body weight, to be constant. However, since in our experiments the dilution factor is large we have calculated the product of dilution factor by acetate fed per unit of body weight.

agent does not occur; if it did, the deuterium concentration should increase with time. It may be inferred that the isotope content of the acetyl group excreted at any time is representative of the metabolic mixture of dietary and endogenous acetate then at the site of acetylation and that the rate of the acetylation reaction is rapid.

The possibility that the production of endogenous acetate is stimulated by a foreign amino acid, such as phenylaminobutyric acid, was tested in Experiment 2 by decreasing the dietary addition of foreign amino acid to about one-fifth of that in other experiments. The product of dilution

TABLE II

Deuterium Concentrations in Acetyl Groups of Acylamino Acids after Simultaneous Feeding to Rats of Sodium Deuterio Acetate and Foreign Amino Compounds for 8 Days

Experiment No.	Deuterio acetate fed per day		Amino acid fed (c)	Deuterium concentration in		Dilution factor $\frac{(b)}{(d)}$	Product, dosage × dilution factor $\frac{(a) \times (b)}{(d)}$
	(a)	(b)		Body water	Acetyl groups (d)		
	mM per 100 gm. body weight	*atom per cent excess deuterium*		*atom per cent excess*	*atom per cent excess*		
1	1.57	6.9	*p*-Aminobenzoic acid	0.02	0.59	11.8	18.5
	1.57	6.9	" "	0.02	0.67	10.4	16.3
	1.57	6.9	" "	0.02	0.60	11.6	18.2
2	1.60	54.1	*dl*-Phenylaminobutyric acid	0.08	4.57	11.8	18.9
	1.60	54.1	" "	0.08	4.58	11.8	18.9
3	1.60	54.1	*l*(+)-Phenylaminobutyric acid	0.09	4.13	13.1	21.0
	1.60	54.1	" "	0.09	4.96	11.0	17.6
4	1.51	54.1	*d*(−)-Phenylaminobutyric acid	0.10	4.99	10.8	16.4
	1.51	54.1	" "	0.10	3.80	14.2	21.4
5	0.91	60.0	Sulfanilamide	0.09	2.92	20.5	18.7
	0.91	60.0	"	0.09	3.67	16.4	14.9

factor by dosage of acetate was not affected. Furthermore, following administration of *p*-aminobenzoic acid or sulfanilamide (Experiments 1 and 5, Table II), which, in contrast to phenylaminobutyric acid, are mainly excreted unchanged or as glycuronides, the observed dilution factors are almost identical.

The dilution of isotope occurring in the acetylation reaction in the presence of dietary deuterio acetate must therefore be the result either of a dilution of the dietary acetate by endogenous acetate or of a formation of acetyl groups from non-acetate precursors. At least part of the isotope

dilution must be caused by acetic acid normally formed in intermediary metabolism, for the excretion of deuterio acetyl groups after the feeding of labeled butyrate, myristic acid, and leucine, described in earlier papers (8, 19), can be explained only on the assumption that these compounds yielded acetic acid on degradation. Evidently, then, acetic acid will arise normally from fatty acids and some amino acids. If deuterio acetate is added to the diet, it will merge with the normal acetic acid and its isotope concentration thus can become diluted prior to the acetylation.

Though the appearance of deuterio acetyl groups is proof of the participation of acetic acid in acetylation reactions, the possibility still exists that part of the acetyl is of different origin. Pyruvic acid has been shown to acetylate choline *in vitro* in the presence of an oxidizing agent (20). A similar mechanism operating *in vivo* would lead to acetyl groups with only normal hydrogen and would lower the isotope content of the excreted acetyl derivative.

A mechanism involving pyruvic acid has been proposed by Knoop (11) and discussed by du Vigneaud *et al.* (15). Our finding that the administration of deuterio alanine and phenylaminobutyric acid results in the excretion of acetyl groups containing a high concentration of deuterium can be interpreted in two ways.[3] Pyruvic acid arising from alanine may have been degraded to acetic acid which in turn was utilized as a source of acetyl. If this were the case, deuterium should be found in all the products which deuterio acetate is known to form; *i.e.* not only in the acetyl group of phenylaminobutyric acid but also in acetylaminobenzoic acid, acetylsulfanilamide, and in cholesterol. The data given in Table III do not support this view. The acetyl groups of aminobenzoic acid and sulfanilamide had a deuterium content only 10 to 20 per cent of the value which should result from complete breakdown of alanine to acetic acid (Table III). Furthermore, alanine is the only compound so far encountered which is an effective source of acetyl groups for phenylaminobutyric acid but fails to give rise to deuterio cholesterol. On the other hand, acetyl groups may arise from alanine (or pyruvic acid) by direct condensation of pyruvate with

[3] We believe that a direct utilization of alanine by transamination with phenylketobutyric acid is not involved, since no significant concentrations of N^{15} were found in the excreted acetylphenylaminobutyric acid when N^{15}-containing alanine was fed. It is generally accepted that a large fraction of dietary alanine is converted to pyruvic acid. Deuterio alanine therefore acts as a source of deuterio pyruvic acid. When deuterio pyruvate was administered as a source of acetyl, the excreted acetyl derivative contained only one-fifth of the deuterium concentration which resulted from the feeding of corresponding amounts of alanine. Sodium pyruvate is a chemically unstable compound and we cannot be certain that all of the pyruvate ingested by the animal reached the liver unchanged. We therefore believe that the results obtained with pyruvate do not invalidate the assumption that alanine acts as a source of acetyl by way of pyruvic acid.

a foreign amino acid and subsequent dehydrogenation of the product (Knoop mechanism). The fact that aromatic amines take up but little acetyl from alanine may be ascribed to their lack of hydrogen at the carbon atom attached to the nitrogen, and their consequent inability to yield imino compounds analogous to imino acids. The possibility that the breakdown of alanine to acetic acid was inhibited by the aromatic amines was tested by simultaneous administration of deuterio alanine, phenylaminobutyric acid, and *p*-aminobenzoic acid. From the mixture of acetyl derivatives

TABLE III

Deuterium Concentrations in Acetyl Groups of Acyl Derivatives and in Cholesterol after Simultaneous Feeding of Deuterio dl-Alanine and Foreign Amines for 8 Days

Experiment No.	Amine fed per 100 gm. of rat weight		Deuterio *dl*-alanine fed		Deuterium concentration in: Body water	Acetyl group	Cholesterol: Liver	Cholesterol: Carcass†
		mg.	*atom per cent excess deuterium**	*mM per 100 gm. rat weight*	*atom per cent excess*	*atom per cent excess*	*atom per cent excess*	*atom per cent excess*
1	*dl*-Phenylaminobutyric	100	60.7	0.84	0.09	1.51	0.021	0.018
	acid	100	60.7	0.84		1.58		
		100	60.7	0.84		1.74		
2	*l*-Phenylaminobutyric acid	50	60.7	0.83	0.08	2.14		
	+ *p*-aminobenzoic acid	50						
3	*p*-Aminobenzoic acid	100	22.0	1.6	0.130	0.25	0.031	
		33	30.0	1.5	0.151	0.36	0.020	0.006
		100	60.7	0.81	0.08	0.18	0.025	0.011
		100	60.7	0.81		0.18		
4	Sulfanilamide	55	60.7	0.91	0.10	0.27	0.024	0.013
		55	60.7	0.91	0.10	0.36		

* Calculated for the hydrogen atoms at the β-carbon atom.

† When equivalent amounts of deuterio acetate containing 54 atom per cent excess D_2 were fed, the carcass cholesterol contained 0.25 atom per cent excess D_2.

excreted in the urine, acetylphenylaminobutyric acid was isolated. Its isotope concentration was not depressed; the inability of alanine to acetylate aromatic amines can therefore not be due to an interference of alanine breakdown by these compounds. It would appear likely that when deuterio alanine is fed the small deuterium concentrations in acetylamino benzoic acid and in acetylsulfanilamide are due to the formation of acetic acid from pyruvic acid at the site of acetylation. The data (Table III) suggest that in the liver this reaction constitutes only a minor pathway of pyruvate metabolism.

The data given in Table II indicate that the product of the dosage of dietary deuterio acetate and the dilution factor varies but little with the nature of the foreign amine administered. The finding that acetic acid is an equally effective acetylating agent in each case suggests that essentially the same mechanism involving direct condensation with acetic acid may be concerned in the acetylation of *d*- and *l*-phenylaminobutyric acids, *p*-aminobenzoic acid, and sulfanilamide. The average value of this product for all experiments with phenylaminobutyric acid is 21.0, as compared to an average of 17.3 for the experiments with sulfanilamide and aminobenzoic acid. As the number of such experiments is small, the difference between these two values is not statistically significant; the results can be regarded merely as concordant with the hypothesis that in the acetylation of α-amino acids only a small part of the isotope dilution is due to the formation of acetyl by a mechanism which does not involve acetic acid.

Acetylation of Optically Active Phenylaminobutyric Acid—Bernhard has observed the utilization of deuterio acetate for the biological acetylation of both *d*- and *l*-hexahydrophenylalanine (21) to yield the acetyl-*l*-amino acid. Under our experimental conditions very nearly the same dilution factors were obtained for the acetyl group of racemic phenylaminobutyric acid and its two optical isomers (see Table II). The mechanism involved in the acetylation of the optically active phenylaminobutyric acids has been studied in great detail by du Vigneaud *et al.* (15), with the aid of heavy nitrogen and deuterium. No decision was possible from the work of these authors as to the nature of the acetylating agent, although the mechanism involving either pyruvic acid as originally suggested by Knoop or acetate appeared to account satisfactorily for the experimental findings. Bernhard's data, as well as our own, definitely establish the ability of acetic acid (either as such or as a biochemically active derivative, such as acetyl phosphate) to acetylate amines.

It seems that the inversion of the unnatural acid is not necessarily linked with acetylation and the process of hydrogenation and dehydrogenation postulated (15) to explain the uptake of deuterium in the α position of a foreign amino acid seems to be incidental. Indeed it is possible for a *d*-amino acid to be acetylated without inversion, as was shown by du Vigneaud, Wood, and Binkley (22) in the case of *p*-bromophenyl-*d*-cysteine. The keto acid formed by deamination of the *d* isomer will be reaminated to the natural amino acid which can then be acetylated by acetic acid in a subsequent reaction. Though our experiments demonstrate that *l*-phenylaminobutyric acid can be acetylated by deuterio alanine, we have no information as to whether the same holds true for the *d* isomer.

Fishman and Cohn (18) have reported that in animals whose body fluids are enriched with D_2O, the administration of either *p*-aminobenzoic acid,

phenylaminobutyric acid, or sulfanilamide leads to the excretion of acetyl derivatives containing from 2 to 3 atoms of deuterium in the acetyl group. In the course of the β oxidation of the fatty acids, acetate becomes available. As the α-hydrogen atoms of β-keto acids are extremely active in exchange reactions, deuterium from the body fluids could readily enter, and in the hydrolysis of the β-keto acid to yield acetic acid, 1 more atom of deuterium could be introduced into the methyl group. Once the acetic acid is formed the deuterium in the methyl group is stably bound. The suggestion that acetic acid is the major source of acetyl groups is therefore entirely consistent with the findings of Fishman and Cohn, whose experiments further indicate that pyruvate cannot be the sole source of acetyl groups, for the methyl group of pyruvate does not exchange its hydrogen atoms readily, as is shown by the study of Reitz on the glucose fermentation in D_2O (23).

Another acetylating agent which may be postulated is acetoacetate. This assumption requires that acetic acid condenses to form acetoacetic acids in which both the carboxyl carbon atom and the β-carbon atom originate from the carboxyl carbon atom of acetic acid. This is necessary, for we have demonstrated that both halves of butyric acid are employed in forming acetyl groups (8). Since such a condensation would involve loss of carbon-bound deuterium, but not of C^{13}, the ratio of the concentration of these isotopes ($D:C^{13}$) in the excreted acetyl group would be different from that in the acetate fed. Since this is not the case, we may exclude acetoacetate as the direct acetylating agent.

We therefore conclude from our data that acetic acid is the only acetylating agent for aromatic amines and the major one for α-amino acids, and that the dilutions observed are the result of a large daily formation of acetic acid. It is permissible to draw quantitative deductions from the results of the isotope dilution technique if the acetylation reaction is shown to involve no dilution other than that by endogenous acetic acid. The data submitted here eliminate a number of other reactions as being responsible for the isotope dilution of the dietary deuterio acetate. If, as appears probable, acetic acid is the sole source of acetyl groups for sulfanilamide and *p*-aminobenzoic acid, the dilution occurring in the acetylation of these aromatic amines may be taken as a direct measure of acetic acid formation by the animal. The existence of two separate acetylating mechanisms for phenylaminobutyric acid, *i.e.* direct acetylation by acetic acid and condensation with pyruvic acid, introduces but little uncertainty into conclusions derived from experiments with this α-amino acid, since acetic acid is quantitatively a much more important source of acetyl groups.

From the dilution factors observed it can be estimated that about 15 to 20 mM (0.9 to 1.2 gm.) of acetic acid are formed daily per 100 gm. of rat tissue. The major portion of the acetic acid probably arises from β

oxidation of fatty acids. This mechanism, potentially, can account for these quantities of acetic acid. A 300 gm. rat contains about 20 gm. of tissue fatty acids, half of which are destroyed and resynthesized in about 9 days (24). Thus, approximately 1.6 gm. of fatty acids are destroyed daily, corresponding to 3.0 gm. of acetic acid daily. We calculate from the data in this paper that a 300 gm. rat would produce about 3 gm. of acetic acid daily. The site of the acetylation reaction is presumed to be the liver (25). If acetic acid were formed in extrahepatic tissues, then it is possible that the dietary deuterio acetate does not merge with all of the acetate produced by the animal. The dilutions observed in the acetyl groups would then represent minimum values of endogenous acetic acid production.

The influence of dietary additions on the acylation of foreign amines, such as sulfanilamide or *p*-aminobenzoic acid, has frequently been investigated by determining the ratio of acetyl derivative to unchanged amine excreted. It appears quite improbable that the balance type of experimentation with intact animals can furnish information on the mechanism of the acetylation reaction. Inspection of such data reveals a very considerable variation of the control values. Moreover, it has been claimed on the basis of such experiments that dietary acetate is incapable of increasing the percentage of acetylation (26, 27). The experiments of Bernhard and those reported here clearly demonstrate, however, that dietary acetate can acetylate amines. We have observed that the absolute amounts of acetyl derivative excreted are independent of the addition of acetate or acetyl precursors. These must be available from normal metabolic reactions in quantities more than adequate for acetylation of the foreign amines. However, when isotopic test substances are employed, no note need be taken of the yield of acetyl derivative, for the appearance of isotope in the acetyl group is evidence of utilization.

Estimation of Cholesterol Formation from Acetate—In one of the experiments in which rats had been given deuterio acetic acid in conjunction with phenylaminobutyric acid (Experiment 2, Table I), cholesterol was isolated separately from the liver, from the remaining internal organs, and from the eviscerated carcass. The sterol samples contained 0.50, 0.45, and 0.25 atom per cent excess, or 27, 24, and 13 per cent respectively of the deuterium concentration in the acetyl group of the acetylphenylaminobutyric acid excreted. If the metabolic mixture of normal and isotopic acetate available for cholesterol formation has the same composition as that used for the acetylation, then at least 27 per cent of all hydrogen atoms of the liver cholesterol had been derived from acetic acid. As in the utilization of each C_2 unit for the sterol structure, 2 hydrogen atoms from the body fluids must be taken up for every 2 from the acetate, at least 54 per cent of the

cholesterol carbon atoms was supplied by acetic acid. The replacement of total body cholesterol in mice by sterol synthesized *de novo* proceeds with a half time of 15 to 25 days (10). Comparable figures for the rat are not available as yet. In the present 8 day experiment in which cholesterol was isolated, twice as much newly formed cholesterol was present in the liver as in the carcass, indicating differences in the rates of cholesterol turnover for different tissues. Nevertheless, it is not certain that replacement of the liver cholesterol by newly synthesized material had approached completion within the 8 day experimental period. Determination of the half life time of liver cholesterol will permit us to estimate more accurately the extent to which acetate serves as a cholesterol precursor.

We are indebted to Mr. M. M. K. Zung for valuable assistance in the course of this work.

SUMMARY

1. The acetylation of foreign amines by acetic acid has been quantitatively investigated in the rat.

2. By employing acetate labeled with C^{13}, as well as deuterium, it has been shown that no loss of deuterium due to exchange reactions occurs in the acetylation reaction.

3. Acetic acid is an effective acetylating agent for *p*-aminobenzoic acid, *d*- and *l*-phenylaminobutyric acids, and sulfanilamide.

4. Evidence is presented to show that acetic acid is the only acetylating agent for the aromatic amines, sulfanilamide and *p*-aminobenzoic acid.

5. Acetic acid is the major source of acetyl groups in the acetylation of phenylaminobutyric acid, which, however, can also be acetylated by a mechanism probably involving pyruvic acid.

6. From the dilution of the acetate fed it is calculated that 15 to 20 mM of acetic acid are formed daily per 100 gm. of rat tissue. It is suggested that the major part of this acetate arises from the oxidation of fatty acids.

7. It is estimated that at least half of the carbon atoms of cholesterol are derived from acetate.

BIBLIOGRAPHY

1. Bloch, K., and Rittenberg, D., *J. Biol. Chem.*, **145**, 625 (1942).
2. Krebs, H. A., and Johnson, W. A., *Biochem. J.*, **31**, 640 (1937).
3. Buchanan, J. M., Hastings, A. B., and Nesbett, F. B., *J. Biol. Chem.*, **150**, 413 (1943).
4. Loeb, A., *Biochem. Z.*, **47**, 118 (1912).
5. Swendseid, M. E., Barnes, R. H., Hemingway, A., and Nier, A. O., *J. Biol. Chem.*, **142**, 47 (1942).
6. Rittenberg, D., and Bloch, K., *J. Biol. Chem.*, **154**, 311 (1944).

7. Bernhard, K., *Z. physiol. Chem.*, **267,** 91 (1940).
8. Bloch, K., and Rittenberg, D., *J. Biol. Chem.*, **155,** 243 (1944).
9. Knorr, L., *Ber. chem. Ges.*, **17,** 1635 (1884).
10. Rittenberg, D., and Schoenheimer, R., *J. Biol. Chem.*, **121,** 235 (1937).
11. Knoop, F., *Beitr. chem. Physiol. u. Path.*, **6,** 150 (1905).
12. Halford, J. O., and Anderson, C. L., *J. Am. Chem. Soc.*, **58,** 736 (1936).
13. Knoop, F., and Oesterlin, H., *Z. physiol. Chem.*, **170,** 186 (1927). Rittenberg, D., Ratner, S., and Hoberman, H. D., *J. Am. Chem. Soc.*, **62,** 2249 (1940).
14. du Vigneaud, V., and Irish, O. J., *J. Biol. Chem.*, **122,** 349 (1937–38).
15. du Vigneaud, V., Cohn, M., Brown, G. B., Irish, O. J., Schoenheimer, R., and Rittenberg, D., *J. Biol. Chem.*, **131,** 273 (1939).
16. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **15,** 311 (1913).
17. Bok, L. D. C., and Geib, K. H., *Z. phys. Chem., Abt. A*, **183,** 353 (1939).
18. Fishman, W. H., and Cohn, M., *J. Biol. Chem.*, **148,** 619 (1943).
19. Bloch, K., *J. Biol. Chem.*, **155,** 255 (1944).
20. Baer, E., *J. Biol. Chem.*, **146,** 391 (1942).
21. Bernhard, K., and Steinhauser, H., *Z. physiol. Chem.*, **273,** 31 (1942).
22. du Vigneaud, V., Wood, J. L., and Binkley, F., *J. Biol. Chem.*, **138,** 369 (1941).
23. Reitz, O., *Z. phys. Chem., Abt. A*, **175,** 257 (1935).
24. Bernhard, K., and Bullet, F., *Helv. chim. acta*, **26,** 1185 (1943).
25. Klein, J. R., and Harris, J. S., *J. Biol. Chem.*, **124,** 613 (1938).
26. Doisy, E. A., Jr., and Westerfeld, W. W., *J. Biol. Chem.*, **149,** 229 (1943).
27. Martin, G. J., and Rennebaum, E. H., *J. Biol. Chem.*, **151,** 417 (1943).

SOME BIOLOGICAL EFFECTS PRODUCED BY α-TOCOPHEROL QUINONE

By D. W. WOOLLEY*

(*From the Laboratories of The Rockefeller Institute for Medical Research, New York*)

(Received for publication, March 1, 1945)

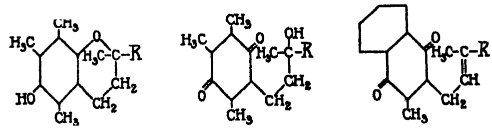
During a study of inhibitory structural analogues of metabolites (1), it has been observed that the administration of α-tocopherol quinone to pregnant mice resulted in the resorptive interruption of pregnancy. Since various signs associated with deficiencies of several vitamins have been called forth in animals by the feeding of compounds analogous in structure to the vitamins concerned with these manifestations (1–5), investigations have been in progress to determine more about such phenomena. In particular, the possible application of such results to pharmacology has been examined. The present findings ensued when attempts were made to produce the signs of tocopherol deficiency by administration of a suitable structural analogue of this vitamin.

It is known that in mature rats or mice tocopherol deficiency manifests itself to a large extent by death and resorption of the embryos during the latter part of the gestation period. Such signs as muscular dystrophy or death are seen only after prolonged deprivation, and usually not until the second generation in these species. This restriction of the signs of deficiency to pregnant animals prompted the present work. It was found that α-tocopherol quinone called forth in mice manifestations related to those seen in tocopherol deficiency. Equivalent doses of the compound were without detectable effect on non-pregnant mice, although if the dose was increased sufficiently some evidence of toxicity was seen in such animals. No muscular dystrophy was observed following use of the agent. Furthermore, no permanent damage was done to the ability to reproduce, since animals which had been through a resorption produced litters when they were returned to stock rations and remated.

Although the signs elicited by tocopherol quinone resembled in some respects those seen in tocopherol deficiency, they were not prevented by α-tocopherol acetate. It was then observed that resorption was usually accompanied by excessive vaginal bleeding. When attempts to prevent this hemorrhage by administration of vitamin K were made, it was found that both the hemorrhage and the resorption were overcome by this vitamin. Inspection of the formulae of the three substances under consideration revealed a structural analogy among all three. The action of

* With the technical assistance of M. L. Collyer.

the quinone, however, differed from that of such a structurally related antagonist of vitamin K as 3,3′-methylenebis(4-hydroxycoumarin) in its



α-tocopherol α-tocopherol quinone Vitamin K_1

$R = C_{16}H_{33}$

selective effect on pregnant mice, in the fact that the hemorrhages were confined to the reproductive system, and that no marked prolongation of prothrombin time was observed, following the use of the quinone.

A few other structural analogues of α-tocopherol were synthesized and tested for biological effect, but these compounds were inactive under the conditions tried.

EXPERIMENTAL

Materials—Synthetic *dl*-α-tocopherol and its acetate were used. α-Tocopherol quinone was made by oxidation of α-tocopherol with gold chloride according to the method of Karrer and Geiger (6). "Demethyltocopherol," that is α-tocopherol without the three —CH_3 groups on the benzene ring, was prepared from phytol[1] and hydroquinone monobenzoate. Several attempts were made to condense trichlorohydroquinone with either phytyl bromide or phytol, but the unchanged trichlorohydroquinone was always recovered in good yield from the reaction mixture. It would be of interest to assay the desired condensation product, if it could be obtained, since it would bear the same relationship to α-tocopherol as the successful antagonist, 6,7-dichloro-9-ribityl-isoalloxazine, bears to riboflavin (7). Similarly, no success was encountered in attempts to condense allyl bromide with trichlorohydroquinone.

Assay Procedure—Adult female mice from the stock colony were caged in groups of four in metal boxes with wood shavings on the bottoms. One male was placed in each box before the females were admitted. Usually both males and females were of proved fertility, but occasionally virgin females were employed. Water and food were supplied *ad libitum*. The ration was composed of sucrose 60 parts, vitamin-free casein 20 parts, salts (8) 5 parts, dried brewers' yeast 5 parts, lard 5 parts, cod liver oil 4 parts, and the following crystalline vitamins in mg. per 100 gm. of ration:

[1] We wish to thank Dr. H. M. Wuest of Hoffmann-La Roche, Inc., for generous gifts of phytol and phytyl bromide. We are also indebted to Dr. K. C. D. Hickman of Distillation Products, Inc., for quantities of natural tocopherol concentrates used in several preliminary synthetic investigations.

thiamine 0.2, riboflavin 0.5, pyridoxine 0.2, calcium pantothenate 2.0, nicotinic acid 10, inositol 100, and choline 100. On the 5th day of pregnancy oral administration of the compounds to be tested was begun. The females were dosed daily until parturition or resorption occurred. The animals were weighed once weekly for the first 2 weeks. They were then caged separately and weighed daily until the end of the experiment. All animals except those upon which autopsies were performed at the time of resorption were maintained for 3 weeks after they were isolated in individual cages.

Effect of dl-α-Tocopherol Quinone on Pregnant Mice—The daily oral administration of 100 mg. of *dl-α*-tocopherol quinone caused no detectable signs of disease in pregnant mice until the 3rd week of gestation. Then the rapid increase in weight was suddenly arrested at the 14th to 19th day, and about a day later extensive vaginal bleeding occurred. This sometimes continued intermittently for a day or two, but in many cases there appeared to be just one hemorrhage. The loss of blood was usually so extensive that the animals became markedly anemic. A few died 2 or 3 days after the first hemorrhage, due to excessive loss of blood. Precipitous loss of weight occurred usually beginning 1 day after the first hemorrhage. When autopsies were performed on animals which had begun to lose weight, the embryos appeared as slightly bloody masses usually amorphous unless they were seen at the onset of the changes described. No hemorrhages from other parts of the body were encountered. Following the onset of resorption the weight declined to that in the non-pregnant state, or slightly less, and remained at about this level. The animals appeared to be ill for a few days after the episode, but soon began to eat well, and to recover from the anemia. Two instances were observed in which resorption occurred without hemorrhage. Some data to illustrate the effects of tocopherol quinone are shown in Table I.

Relative Potency of α-Tocopherol Quinone by Oral and Intraperitoneal Routes in Mice—It can be seen from the data in Table I that it required 100 mg. per day by the oral route to achieve the desired effect. To learn whether this large dose might be in part due to the water insolubility and poor absorbability of the compound, the quinone was tested by the intraperitoneal route. It was expected that tocopherol quinone, like many other oily substances, would be absorbed rather slowly from the abdominal cavity, and thus that one single dose would suffice for considerable periods of time. It was found that the colored quinone could still be seen readily in the peritoneal cavity 2 weeks after 400 mg. had been injected. To determine the activity of the quinone by this route in the production of resorptive interruption of pregnancy, a single dose was given intraperitoneally on the 4th or 5th day of pregnancy. As can be seen from the

data in Table I, the compound was much more active by this route than by oral administration.

With a single intraperitoneal dose of 400 mg. no signs were seen for a week following injection. However, during the last week of pregnancy all of the six mice so treated resorbed. Two of these died at the beginning of the last week of gestation without exhibiting hemorrhage externally, but with extensive bleeding into the peritoneal cavity. Towards the end of this last week three of the remaining mice showed signs similar to those described above in animals given the agent orally.[2] These three died from loss of blood. The remaining mouse lost weight but recovered.

With single intraperitoneal injections of 200 mg. of the quinone, results comparable to those obtained with daily oral doses of 50 mg. were found.

TABLE I

Effect of Various Analogues of α-Tocopherol in Pregnant Mice

Compound	Oral dose	Intra-peritoneal dose	No. of animals	No. of litters	Average litter size	No. of deaths
	mg. per day	*mg.**				
None†			33	33	6	0
"Demethyl-tocopherol"	50	0	4	4	4	0
dl-α-Tocopherol quinone	10	0	4	4	7	0
" "	50	0	8	4	6	0
" "	100	0	14	2	3	2
" "	0	200	12	8	5	2
" "	0	400	6	0		5

* This was the total amount of material used per gestation.

† In two experiments the basal animals were dosed orally with 100 mg. of olive oil per mouse per day. This had no adverse effect that was detectable.

It was concluded that intraperitoneal injection allowed greater expression of potency.

Effect of dl-α-Tocopherol Quinone on Non-Pregnant Mice—When non-pregnant female mice were given 100 mg. of the quinone per day for 6 weeks, no signs of disease were observed. When four immature females were given 150 mg. of the quinone per day for 3 weeks, no retardation of growth was encountered. One of these mice died after 2.5 weeks, but autopsy revealed no hemorrhage. Furthermore, the prothrombin time (9) of the remaining three animals did not differ significantly from that of controls not receiving the compound, or of normal mice from the stock

[2] These three mice did not lose blood into the peritoneal cavity, but only externally from the reproductive tract.

colony. It was concluded that tocopherol quinone, even in doses greater than those which were effective in pregnant mice, was relatively innocuous in non-pregnant animals.

Observations on Permanent Damage Caused by α-Tocopherol Quinone—To determine whether the quinone caused permanent damage to the ability of mice to reproduce, the following experiment was performed. Five animals which had resorbed their litters as a result of treatment with 100 mg. of the quinone per day were placed back on the stock ration and remated. All five produced vigorous litters. It was concluded that no damage had been done to the ability to reproduce.

Attempted Reversal of Effects of α-Tocopherol Quinone with α-Tocopherol Acetate—To determine whether or not the effects of α-tocopherol quinone could be prevented by tocopherol, pregnant mice were treated with daily oral doses of 100 mg. of the quinone, and in addition were given massive

TABLE II

Effect of Vitamin E and Vitamin K on Pregnant Mice Given 100 Mg. of dl-α-Tocopherol Quinone Orally

Compound	Oral dose	No. of animals	No. of litters	Average litter size	No. of deaths
	mg. per day				
None		8	0		1
dl-α-Tocopherol acetate	20	4	0		1
" "	50	4	0		0
2-Methyl-1,4-naphthoquinone	0.02	12	12	7	0

doses of *dl*-α-tocopherol acetate daily by mouth. As can be seen from the data in Table II, the vitamin was not able to prevent the manifestations produced by the quinone.

Prevention of Effects of α-Tocopherol Quinone with Vitamin K—When attempts were made by administration of vitamin K to prevent the excessive bleeding which usually accompanied the resorptive termination of pregnancy, it was found that not only the vaginal hemorrhages but also the resorptions were prevented. The data in Table II show that small doses of 2-methyl-1,4-naphthoquinone given along with tocopherol quinone allowed the normal production of young. The vitamin was fed daily as a solution in olive oil.

Failure of 3,3'-Methylenebis(4-hydroxycoumarin) to Cause Interruption of Pregnancy—Since vitamin K reversed the effects of α-tocopherol quinone, and since vitamin K and the quinone are structural analogues, an experi-

ment was performed to determine whether another competitive structural analogue of vitamin K, namely 3,3'-methylenebis(4-hydroxycoumarin), would produce the same result as the quinone. Ten pregnant mice were given daily oral doses of 0.4 mg. each of the coumarin. It was established previously that 1.0 mg. of the coumarin was fatal to all mice tested. One of the pregnant mice developed hemorrhages around the ears, and a second animal died during the 3rd week of the test. The others, as well as the hemorrhagic one, bore living young without apparent difficulty. Therefore, it was concluded that the quinone and the coumarin differed in their action on pregnant mice.

Effect of α-Tocopherol Quinone on Prothrombin Time of Mouse Plasma—In view of the reversal by vitamin K of the action of the quinone, prothrombin times were determined on a number of treated mice as well as on control animals in an effort to learn whether signs of vitamin K deficiency other than hemorrhage were elicited by the quinone. Estimations were made according to the directions of Campbell *et al.* (9). Mice which had received daily oral doses of 100 mg. of the quinone, and which had manifested hemorrhage and resorption a few days before the test, were examined. The average prothrombin time for eight such mice was 31 seconds, while that for nine controls was 29 seconds. Controls did not differ significantly from mice on the stock diet in this respect. The animals were examined 40 hours after they had received their last dose of the quinone.

DISCUSSION

The experiments with α-tocopherol quinone demonstrate once again that it is possible to produce specific pharmacological results by administration of suitable structural analogues of various metabolites. These manifestations can be predicted at least in part by a knowledge of the signs of deficiency of the metabolite concerned. By prior knowledge that tocopherol deficiency in mice or rats resulted in the resorptive interruption of pregnancy, it was possible to proceed to an agent which would bring about a similar result.

The fact that signs superficially resembling those of tocopherol deficiency were observed was of interest when it was found that tocopherol failed to overcome the effect, and that vitamin K was active in this respect. The quinone may be regarded as a structural analogue of both vitamins. Furthermore, Tishler and Evans (10) have examined a compound which had vitamin E activity as well as vitamin K potency. The only sign related to vitamin K deficiency which was produced by the quinone was the hemorrhage. This manifestation was apparently confined to the reproductive system, and was not seen when similar doses of the compound

were given to non-pregnant animals.[3] Moreover, hemorrhage in the reproductive system of avitaminotic rats (11) and in the brains of chickens deficient in vitamin E (12) has been recorded. Finally, although the effects of the quinone were overcome by vitamin K, there was evidence that the mechanisms of action of the agent must not be viewed entirely as the production of vitamin K deficiency. The selectivity for pregnant animals would hardly be anticipated if vitamin K deficiency was the basis of the effect. Furthermore an agent such as 3,3′-methylenebis(4-hydroxycoumarin), which produces signs similar to those of vitamin K deficiency, did not cause fetal resorption.

On the other hand the relationship to tocopherol of the effect of the quinone was obscured by failure of that vitamin to reverse the manifestations. Another difficulty was that muscular dystrophy was not produced by the quinone. This may not be surprising, however, since in mice this sign of tocopherol deficiency is usually not seen until the second generation. It will be of interest to determine whether tocopherol quinone will cause muscular dystrophy in animals such as the rabbit which are more prone to this manifestation of avitaminosis E. It will also be instructive to learn whether the quinone will bring about resorptive interruption of pregnancy in those species in which this sign of tocopherol deficiency has not yet been observed.

Amid such perplexities as these it would seem wise to regard the case of tocopherol quinone as a pharmacological model with relationships to both vitamin E and vitamin K.

SUMMARY

Administration of *dl*-α-tocopherol quinone to pregnant mice caused hemorrhage in the reproductive system and resorptive termination of pregnancy during the last week of gestation. Similar amounts of the compound were without detectable effect on non-pregnant mice. No permanent damage was done to the ability to reproduce. The action of the quinone was not prevented by large doses of α-tocopherol acetate, but was negated by small amounts of 2-methyl-1,4-naphthoquinone (vitamin K). The quinone was viewed as a structural analogue of both vitamin E and vitamin K. 3,3′-Methylenebis(4-hydroxycoumarin), which caused signs (reversible by vitamin K) similar to those seen in vitamin K deficiency, did not produce resorption or vaginal hemorrhage in pregnant mice. The quinone was much more effective when given intraperitoneally than when fed. Certain implications of these experiments in pharmacology were indicated.

[3] It may be that the restriction of hemorrhage to pregnant animals is merely a reflection of the mechanical stress placed on a slightly K-avitaminotic organism during the latter part of the gestation period.

BIBLIOGRAPHY

1. Woolley, D. W., *Science*, **100**, 749 (1944).
2. Woolley, D. W., and White, A. G. C., *J. Biol. Chem.*, **149**, 285 (1943).
3. Woolley, D. W., and Krampitz, L. O., *J. Exp. Med.*, **78**, 333 (1943).
4. Emerson, G. A., and Tishler, M., *Proc. Soc. Exp. Biol. and Med.*, **55**, 184 (1944).
5. Woolley D. W., *J. Biol. Chem.*, **154**, 31 (1944); **157**, 455 (1945).
6. Karrer, P., and Geiger, A., *Helv. chim. acta*, **23**, 455 (1940).
7. Kuhn, R., Weygand, F., and Möller, E. F., *Ber. chem. Ges.*, **76**, 1044 (1943).
8. Phillips, P. H., and Hart, E. B., *J. Biol. Chem.*, **109**, 657 (1935).
9. Campbell, H. A., Smith, W. K., Roberts, W. L., and Link, K. P., *J. Biol. Chem.*, **136**, 47 (1940); **138**, 1 (1941).
10. Tishler, M., and Evans, H. M., *J. Biol. Chem.*, **139**, 241 (1941).
11. Mason, K. E., Essays in biology (in honor of H. M. Evans), Berkeley, 401 (1943).
12. Pappenheimer, A. M., and Goettsch, M., *J. Exp. Med.*, **53**, 11 (1931).

PENITRINIC ACID, A NEW PIGMENT FROM PENICILLIUM NOTATUM

BY FRANK H. STODOLA, JACQUES L. WACHTEL, ANDREW J. MOYER, AND ROBERT D. COGHILL

(*From the Fermentation Division, Northern Regional Research Laboratory,** *Peoria, Illinois*)

(Received for publication, March 13, 1945)

During the purification of penicillin, a new pigment has been isolated from *Penicillium notatum* culture liquors, and some observations have been made on its chemical structure. It is an unusual mold pigment, since it contains nitrogen. The presence of this element and the source and acidic nature of the pigment have been indicated by giving this compound the name "penitrinic acid."

The pigment was isolated in the crude state from the eluates of an alumina column used to remove penicillin from protein-free culture liquors. Several recrystallizations gave pale yellow crystals which had a melting range of 217–223° (with decomposition), showed a composition of $C_{15}H_{17}O_6N$ by analysis, and exhibited an unusually high specific rotation (−423° or −549°, depending on the solvent). Electrometric titration showed that penitrinic acid is approximately as strong as benzoic acid.

Like the pigment citromycetin, obtained by Hetherington and Raistrick (1) from a species of *Penicillium*, penitrinic acid can be decarboxylated by boiling with 2 N sulfuric acid. The product is an optically inactive, yellow crystalline compound having the formula $C_{14}H_{17}O_3N$ (m.p. 171–172°). The name "α-penitrin" has been selected for this compound.

Penitrinic acid can be decarboxylated also in alkaline solution to an optically inactive, yellow crystalline product, "β-penitrin" (m.p. 204–205°), which is isomeric with α-penitrin. Alkali solubility tests and color reactions indicated that these degradation products are phenols. The two isomers showed almost identical ultraviolet absorption spectra (Fig. 1). Some chemical differences between the compounds have been observed, however, in their behavior with ferric chloride and with mercuric nitrate.

Since citromycetin has been shown to have a chromone nucleus (1), a similar structure was considered for penitrinic acid. Both substances arise from *Penicillia* and have some reactions in common. However, attempts to show the presence of a chromone ring in penitrinic acid through alkaline fusion have not yet led to any definite products.

* One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture.

The nature of the nitrogen is still obscure. All that is known at present is that it is not basic and that on steam distillation with 1 N NaOH for 30 minutes only about a quarter of the nitrogen appears in the distillate. The complete loss of optical activity on decarboxylation suggests that the carboxyl group of penitrinic acid is not on a benzene ring but rather on an asymmetric carbon atom elsewhere in the molecule.

At a level of 0.6 mg. per cc., penitrinic acid showed no antibiotic activity against *Staphylococcus aureus* by the cylinder method of assay as described by Schmidt and Moyer (2).

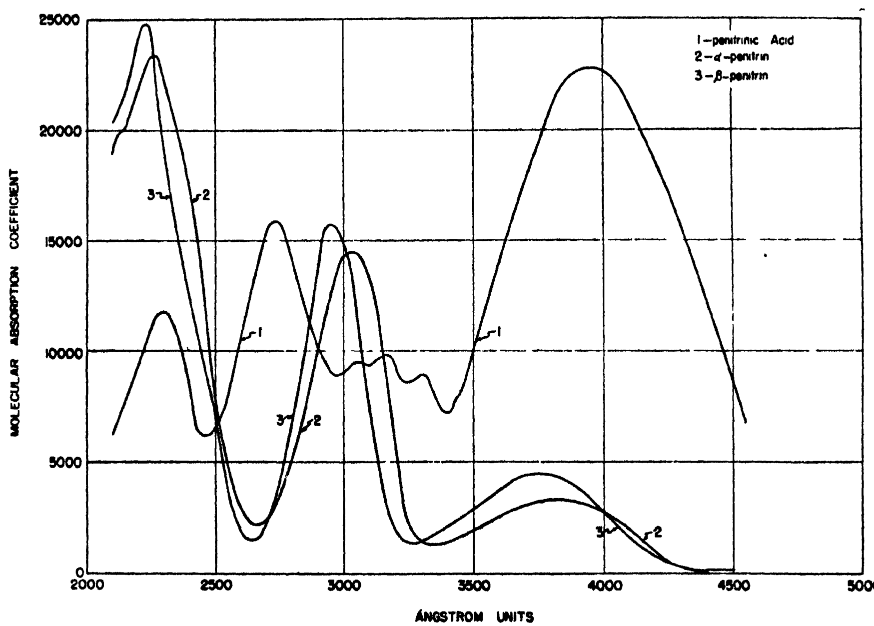


FIG. 1. Ultraviolet absorption spectra of penitrinic acid and α- and β-penitrin

EXPERIMENTAL

Penitrinic Acid—The *Penicillium notatum*, Northern Regional Research Laboratory No. 1249.B21, was grown from 7 to 10 days in Fernbach flasks on a medium containing salts, corn steep liquor, and sugars. After the mold mat was removed by filtration through cloth, the culture liquor was freed of protein to avoid formation of emulsions on solvent extraction. Two methods were used for removal of protein. In the first, proteins were precipitated by tannic acid; in the second, the precipitant was acetone, which was removed by concentration *in vacuo* after filtration. The filtrate was extracted three times with ether to remove pigments. The

ether solution was then applied to a column of acid-treated (to pH 4.5) Brockmann alumina. On development of the column with 75:25 ether-ethyl acetate, orange-colored percolates were obtained. The first colored solution was contaminated with considerable oil; later fractions, however, yielded crude crystalline penitrinic acid on concentration. The pure acid was obtained by repeated crystallization from isopropyl alcohol, from which it separated in the form of pale yellow bars.

Analysis—$C_{15}H_{17}O_5N$. Calculated, C 61.84, H 5.88, N 4.80; found (after 1 hour's drying at 80° under 2 mm. pressure), C 61.8, 61.9, H 5.95, 6.02, N 4.77, 4.83. (The nitrogen values were obtained by micro-Kjeldahl determinations in which 6 hour digestions were used. Dumas values were consistently low.)

$[\alpha]_D^{23} = -549°$ (17.2 mg. in 1.276 cc. dimethyl formamide)

$[\alpha]_D^{23} = -423°$ (2.70 mg. in 1.30 cc. acetone)

Rast molecular weight in camphor, 321 (calculated, 291)

In a capillary tube, the pure pigment decomposed at 217–223° with evolution of gas and the appearance of a red color. Methoxyl groups were absent, according to the Zeisel determination; sodium fusion tests for halogens and sulfur were likewise negative. The pigment is almost insoluble in water, difficultly soluble in ether, methanol, and ethanol, fairly soluble in acetone, and very soluble in dimethyl formamide. It dissolves in sodium bicarbonate solution to give a bright yellow color. Addition of sodium hydroxide causes no change in color; acidification precipitates crystalline penitrinic acid.

Potentiometric titration of the pigment in aqueous acetone showed the presence of a carboxyl group of pK 6.4 and indicated an equivalent weight of 288 (calculated, 291).

With ferric chloride in 50 per cent alcohol, penitrinic acid develops an intense greenish brown color. In the same solvent, mercuric nitrate gives with the acid a voluminous white precipitate soluble in acetic acid.

α-Penitrin—For decarboxylation, 475 mg. of penitrinic acid were refluxed under nitrogen for 16 hours with 50 cc. of 2 N sulfuric acid. An ether extraction was made, and the ether was washed twice with sodium bicarbonate solution to remove unchanged penitrinic acid. The ether solution on concentration yielded 290 mg. of yellow crystals. Repeated recrystallization from aqueous acetone and from acetone-petroleum ether gave 107 mg. of pale yellow needles with a melting point of 171–172° (darkening, but no evolution of gas). β-Penitrin, which is formed in smaller amount at the same time, was not detectable in x-ray diffraction patterns obtained from our best samples.

α-Penitrin appears to be a phenol, since it is soluble in sodium hydroxide and gives momentarily an almost black solution with ferric chloride. It gives a deep blue color with phosphomolybdic acid when ammonia is

added. With mercuric nitrate in 50 per cent alcohol, α-penitrin gives a white precipitate soluble in acetic acid. Unlike penitrinic acid, α-penitrin is optically inactive.

Analysis—$C_{14}H_{17}O_3N$. Calculated, C 67.99, H 6.93, N 5.66; found (after 1.5 hours drying at 100° under 2 mm. pressure), C 68.1, 68.2, H 6.80, 6.80, N 5.49 (Dumas), 5.67 (Kjeldahl)

β-Penitrin—Penitrinic acid (816 mg.) was dissolved in 58 cc. of 0.513 N potassium hydroxide (10.6 equivalents of alkali per mole of compound), and the solution was heated at 60° for 4 hours. The odor of ammonia was detectable in 5 minutes. The brown solution was cooled and carbon dioxide was bubbled through it. The yellow crystals which precipitated were separated (weight 350 mg.) and were found to melt in the range 170–190°. Two crystallizations from acetone-petroleum ether gave 72 mg. of very pale yellow needles, which melted at 204–205° to a dark melt, without evolution of gas.

Unlike α-penitrin, the alkaline hydrolysis product gives only a light orange color with ferric chloride and *no* precipitate with mercuric nitrate in 50 per cent alcohol solution. With phosphomolybdic acid, β-penitrin gives a blue color on addition of ammonia. It is optically inactive.

Analysis—$C_{14}H_{17}O_3N$. Calculated, C 67.99, H 6.93, N 5.66; found (after 1 hour's drying at 80° under 2 mm. pressure), C 68.1, H 6.97, N 5.59 (Dumas), 5.66 (Kjeldahl)

This investigation of penitrinic acid was carried out during our early work on the isolation of penicillin, when it appeared that a pigment might be a part of the penicillin molecule. Since such a relationship does not exist, we are not now in a position to continue our study of this compound.

We are indebted to R. T. Milner, E. H. Melvin, and D. MacMillan of the Analytical and Physical Chemical Division of this Laboratory for the absorption spectra determinations and electrometric titrations. The microanalyses were made by C. H. Van Etten of the same Division.

SUMMARY

A pigment, penitrinic acid, of composition $C_{15}H_{17}O_5N$, has been isolated from *Penicillium notatum* culture liquors. Acid hydrolysis decarboxylates the compound to α-penitrin, $C_{14}H_{17}O_3N$. An isomeric product, β-penitrin, results from alkaline hydrolysis.

BIBLIOGRAPHY

1. Hetherington, A. C., and Raistrick, H., *Phil. Tr. Roy. Soc. London, Series B*, **220**, 209 (1931).
2. Schmidt, W. H., and Moyer, A. J., *J. Bact.*, **47**, 199 (1944).

THE BIOCHEMISTRY OF THE MALARIA PARASITE*

II. GLYCOLYSIS IN CELL-FREE PREPARATIONS OF THE MALARIA PARASITE

By JOHN F. SPECK and E. A. EVANS, Jr.

(*From the Department of Biochemistry of the University of Chicago, Chicago*)

(Received for publication, February 15, 1945)

The formation of lactic acid from glucose is an important reaction in the carbohydrate metabolism of the malaria parasite. Lactic acid appears to be the sole product of glucose utilization by the parasite under anaerobic conditions and also accumulates, as an intermediate, in the oxidation of glucose to carbon dioxide and water in the presence of oxygen (1, 2).

The present report comprises a study of the mechanism of lactic acid formation from glucose by the malaria parasite *Plasmodium gallinaceum*. It has been possible to prepare, for the first time, cell-free enzyme extracts from the parasite capable of the *in vitro* conversion of glucose to lactic acid. These extracts were found to phosphorylate glucose by a transfer of phosphate from adenosine triphosphate and to catalyze the oxidation-reduction between 3-phosphoglyceraldehyde and pyruvic acid.

Methods and Materials

Inorganic phosphate was determined by the method of Gomori (3) on trichloroacetic acid filtrates.

Adenosine triphosphate was isolated from rabbit muscle according to Needham (4). Fructose-1,6-diphosphate was separated from the products of yeast fermentation as the barium salt and purified as the acid barium salt (5). Diphosphopyridine nucleotide was isolated from yeast by the method of Williamson and Green (6).

Other matters of experimental procedure are described later.

Enzyme Preparations

Two types of enzyme preparations were employed in studying the glycolysis of *Plasmodium gallinaceum*. The first type, called a hemolysate, was prepared by treating parasitized red blood cells with water and then centrifuging off the cellular material. This procedure completely laked

* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Chicago. This work was also supported in part by grants from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago. For the first paper in this series, see Silverman, Ceithaml, Taliaferro, and Evans (1).

the red cells and partially disrupted the parasites also. Therefore, the hemolysate contained the glycolytic enzymes from both the red cells and the parasites. Although the greater part of the total enzyme activity was due to the enzymes from the parasites, it was necessary to run control experiments with hemolysates of normal red cells. The second type of preparation, called an extract, was made by laking or saponizing parasitized red cells, washing the liberated parasites free from hemoglobin, and then grinding this parasite material and extracting the enzymes. Since the washing removed the glycolytic enzymes of the red cells, control experiments with preparations from normal red blood cells were not necessary.

The red blood cells of chickens infected with *Plasmodium gallinaceum*, strain 8-A, as designated by the Committee on Terminology of Strains of Avian Malaria of the American Society of Parasitologists, were used in making enzyme preparations.[1] Blood was drawn by heart puncture at the height of parasitemia, about 4 days after inoculation of the chickens with infected blood. At this time 60 to 90 per cent of the red cells contained one or more parasites. Clotting was prevented by adding 1/10 volume of 10 per cent sodium citrate to the blood.

Hemolysates—Hemolysates prepared in a manner similar to that described by Meyerhof (7) in his work on glycolysis in erythrocytes were used to study the phosphorylation of glucose. The citrated blood was centrifuged, and the cells were washed three times by suspending them in 4 volumes of 0.9 per cent sodium chloride and centrifuging again. The washed cells were treated with 2 volumes of distilled water and allowed to hemolyze for 15 minutes with occasional shaking. Then sufficient 7 per cent sodium chloride (1/10 volume) was added to restore the salt concentration to about 0.9 per cent, and the solid material was centrifuged off vigorously. The supernatant solution, which contained large amounts of hemoglobin but was perfectly clear, was used as the enzyme. All operations were carried out in a cold room at 0°. The concentration of the hemolysate was calculated in terms of the volume of packed red blood cells used in its preparation.

Extracts—Extracts of parasite material were employed in studying the oxidation-reduction reactions. The citrated blood was centrifuged, and the cells were washed twice by suspending them in 4 volumes of calcium-free phosphate-saline (8) containing 0.1 per cent glucose. The cells were then suspended in 4 volumes of the same medium and treated with sufficient 4 per cent saponin in phosphate-saline (1/40 volume) to make the final concentration of saponin about 0.1 per cent. These conditions of saponization are similar to those described by Christophers and Fulton (9). After the saponin had been thoroughly mixed with the red cell suspension,

[1] We are greatly indebted to Mr. William Cantrell of the Department of Bacteriology and Parasitology for providing us with the infected birds.

the solid material was centrifuged down and washed twice by suspending in 4 volumes of phosphate-saline-glucose and twice in 0.9 per cent sodium chloride containing 0.1 per cent glucose. This washing removed nearly all the hemoglobin and therefore probably removed the extranuclear enzymes of the red cells also. The washed parasite material was dark gray-brown in color and viscous in consistency; it contained parasites and some white cells and red cell nuclei. This material was ground with an equal volume of powdered Pyrex glass in a mortar or in a bacterial mill (10). 1 volume of water was added to the paste, and after vigorous centrifugation a brown, turbid, cell-free extract was obtained. All steps of the preparation were carried out in a cold room at 0°.

The parasites may also be released from the red blood cells by hemolysis. The procedure used was that described above, except that the washings were carried out with phosphate-saline-glucose and only $1\frac{1}{2}$ volumes of water were used for the hemolysis. This procedure certainly caused some cytolysis of the parasites, since the clear supernatant obtained by centrifuging down the hemolyzed cells was more active in phosphorylating glucose than similar hemolysates prepared from normal chicken red cells. Still, if the solid residue was washed free from hemoglobin, ground with powdered glass, and extracted with water, a solution of enzymes capable of catalyzing the oxidation-reduction reactions was obtained.

Omission of glucose from the wash solutions usually gave extracts of low enzymatic activity. Loss of activity was rapid even at 0°. Extracts prepared in the same way from unparasitized red cells always showed less than 10 per cent of the enzymatic activity usually found in extracts made from parasitized red cells.

Results

Phosphorylation of Glucose

In yeast glucose is phosphorylated by a transfer of phosphate from adenosine triphosphate to glucose, with the formation of glucose-6-phosphate (11) (Equation 1). This reaction is catalyzed by the enzyme hexokinase.

$$\underset{\text{Glucose}}{C_6H_{12}O_6} + \underset{\text{Adenosine triphosphate}}{\text{adenosine}-O-\underset{\underset{O^-}{|}}{\overset{\overset{O}{\|}}{P}}-O-\underset{\underset{O^-}{|}}{\overset{\overset{O}{\|}}{P}}-O-\underset{\underset{O^-}{|}}{\overset{\overset{O}{\|}}{P}}-O^-} \longrightarrow$$

$$\underset{\text{Glucose-6-phosphate}}{C_6H_{11}O_6PO_3^{=}} + H^+ + \underset{\text{Adenosine diphosphate}}{\text{adenosine}-O-\underset{\underset{O^-}{|}}{\overset{\overset{O}{\|}}{P}}-O-\underset{\underset{O^-}{|}}{\overset{\overset{O}{\|}}{P}}-O^-} \qquad (1)$$

Most of the subsequent steps in the glycolytic breakdown of glucose-6-phosphate are the same in yeast and in muscle. The enzyme phosphohexoisomerase brings about the rearrangement of glucose-6-phosphate to fructose-6-phosphate (12). In the presence of the enzyme phosphohexokinase, fructose-6-phosphate is converted to fructose-1,6-diphosphate by a second transfer of phosphate from adenosine triphosphate (13) (Equation 2).

$$\underset{\text{Fructose-6-phosphate}}{C_6H_{11}O_6PO_3^{=}} + \underset{\text{Adenosine triphosphate}}{\text{adenosine}-O-\overset{\overset{\displaystyle O}{\|}}{\underset{\underset{\displaystyle O^-}{|}}{P}}-O-\overset{\overset{\displaystyle O}{\|}}{\underset{\underset{\displaystyle O^-}{|}}{P}}-O-\overset{\overset{\displaystyle O}{\|}}{\underset{\underset{\displaystyle O^-}{|}}{P}}-O^-} \rightarrow$$

$$\underset{\text{Fructose-1,6-diphosphate}}{C_6H_{10}O_6(PO_3^{=})_2} + H^+ + \underset{\text{Adenosine diphosphate}}{\text{adenosine}-O-\overset{\overset{\displaystyle O}{\|}}{\underset{\underset{\displaystyle O^-}{|}}{P}}-O-\overset{\overset{\displaystyle O}{\|}}{\underset{\underset{\displaystyle O^-}{|}}{P}}-O^-} \qquad (2)$$

Magnesium or manganous ions are necessary as cofactors for these phosphate transfer reactions.

Reactions of Glucose Phosphorylation—The phosphorylation of glucose by the malaria parasite was studied in hemolysates of parasitized red blood cells, and control experiments were carried out with hemolysates of normal chicken red cells. Qualitatively the reactions occurring were the same in hemolysates from both normal and parasitized red cells. Both preparations contained the enzyme adenosinetriphosphatase, which hydrolyzes adenosine triphosphate to adenosine diphosphate or adenylic acid and inorganic phosphate. This enzyme could largely be inhibited by the addition of fluoride. In the presence of fluoride and magnesium ions, the hemolysates catalyzed a rapid transfer of phosphate from adenosine triphosphate to glucose. Analyses for the pyrophosphate groups of adenosine triphosphate (phosphate liberated by hydrolysis with 1 N H_2SO_4 for 10 minutes at 100° (14)) indicated that these labile phosphate groups disappeared and a more stable phosphate compound was formed. Fructose analyses by the method of Roe (15) suggested that the product was fructose-1,6-diphosphate. The reaction stopped when one of the labile phosphate groups of adenosine triphosphate had been transferred to glucose. There was no relationship between the percentage of red cells infected and the enzymatic activity of the hemolysate, within the range of 60 to 90 per cent infection.

Manometric Experiments on Glucose Phosphorylation—As Equations 1 and 2 indicate, the transfer of phosphate from adenosine triphosphate to a hydroxyl group results in the formation of acid. Therefore this reaction can conveniently be studied by employing a bicarbonate buffer in the test system and measuring the evolution of carbon dioxide by the Warburg manometric technique (11). In the case of red blood cell hemolysates, acid formation which occurred in the absence of glucose was the result of hydrolysis of the adenosine triphosphate and is referred to as "adenosinetriphosphatase activity." Acid formation which occurred in the presence of glucose was the result of transfer of phosphate from the adenosine triphosphate to glucose, along with some hydrolysis of the adenosine triphosphate;

TABLE I

Adenosinetriphosphatase and Hexokinase Activity in Red Blood Cell Hemolysates

The samples contained 0.028 M $NaHCO_3$, 0.004 M $MgSO_4$, 0.028 M KF, 0.012 M glucose (none when adenosinetriphosphatase activity was being measured), 0.0036 M adenosine triphosphate (tipped in from side arm after equilibration), and 1.4 cc. of hemolysate of normal chicken red cells (equivalent to about 0.36 cc. of cells) or 0.7 cc. of hemolysate of parasitized red cells (equivalent to about 0.21 cc. of cells), in a total volume of 2.5 cc. Warburg manometers; gas phase, 5 per cent CO_2-95 per cent N_2; temperature 39°. The retention factor ((observed CO_2)/(true CO_2)) was about 0.7 for mixtures with hemolysates of normal red cells and 0.85 for mixtures with hemolysates of parasitized red cells. Activities are expressed in microliters of CO_2 evolved per cc. of red cells per hour, corrected for retention. The figures in parentheses show the range of values.

Normal red cells			Parasitized red cells		
No. of samples	Adenosine-triphosphatase	Hexokinase	No. of samples	Adenosine-triphosphatase	Hexokinase
6	56 (31–103)	378 (290–591)	12	76 (44–102)	1630 (890–2270)

it is termed "hexokinase activity." Since the hemolysates contained hemoglobin in high concentrations and since different volumes of hemolysate were used in experiments with normal and parasitized material, it was necessary to correct the observed gas evolution for retention of carbon dioxide by the buffering action of the protein, in order to obtain comparable results.

The results of experiments on the adenosinetriphosphatase and the hexokinase activities of hemolysates of normal and parasitized chicken red cells are collected in Table I. The much greater hexokinase activity found in hemolysates of parasitized erythrocytes indicates that the parasites contain considerable quantities of the enzymes hexokinase and phosphohexokinase, which are released by cytolysis. The small difference in adenosinetriphos-

phatase activity between hemolysates of normal and parasitized red cells shows that most of this enzyme present in the hemolysates was derived from the red cells and not from the parasites.

Glucose Phosphorylation in Parasite Extracts—Extracts of parasite material, when tested in the manometric system for studying the phosphorylation of glucose, showed little activity aside from that caused by the enzyme adenosinetriphosphatase. This interfering enzyme was not inhibited by fluoride. Nevertheless, by the use of a different technique it was possible to show that the extracts were able to phosphorylate glucose. This reaction results in the formation of compounds giving a positive test for fructose in the Seliwanoff reaction (fructose-6-phosphate and fructose-1,6-diphosphate). Extracts of parasite material were able to convert glucose to "fructose," and the reaction could be followed by quantitative determinations of fructose made according to Roe (15). Since this conversion was greatly accelerated by the addition of adenosine triphosphate, it undoubtedly involved phosphorylation of glucose by the reactions more clearly demonstrated by the use of hemolysates.

These experiments show that the malaria parasite initiates the breakdown of glucose by phosphorylation in the manner known to occur in yeast extracts.

Oxidation-Reduction Reactions

Muscle and yeast extracts contain an enzyme, aldolase, which catalyzes the splitting of fructose-1,6-diphosphate, formed by the reactions described above, into 2 molecules of triose phosphate, 3-phosphoglyceraldehyde and dihydroxyacetone phosphate (16, 17) (Equation 3). The next step in glycolysis in these extracts is the oxidation of 3-phosphoglyceraldehyde

$$\text{Fructose-1,6-diphosphate} \rightleftharpoons \text{3-phosphoglyceraldehyde} + \text{dihydroxyacetone phosphate} \quad (3)$$

by diphosphopyridine nucleotide (DPN) in the manner represented by Equation 4 (18); the enzyme catalyzing this reaction is 3-phosphoglyceraldehyde dehydrogenase. This equilibrium reaction goes in the forward

$$\text{3-Phosphoglyceraldehyde} + \text{phosphate} + \text{DPN}^+ \rightleftharpoons \text{1,3-diphosphoglyceric acid} + \text{DPNH} + \text{H}^+ \quad (4)$$

direction because of the further reaction of the 1,3-diphosphoglyceric acid and because of the reoxidation of the reduced DPN (DPNH) by pyruvate (formed from the 1,3-diphosphoglyceric acid) (Equation 5); the enzyme which catalyzes this oxidation, lactic dehydrogenase, is present in muscle extracts. One phosphate group of the 1,3-diphosphoglyceric

acid is transferred to adenosine diphosphate in the presence of the appro-

$$\text{DPNH} + \text{H}^+ + \text{pyruvate} \rightleftharpoons \text{DPN}^+ + \text{lactate} \qquad (5)$$

priate enzyme (18, 19) (Equation 6). The 1,3-diphosphoglyceric acid

$$\text{1,3-Diphosphoglyceric acid} + \text{adenosine diphosphate} \rightleftharpoons \text{3-phosphoglycerate} + \text{adenosine triphosphate} \qquad (6)$$

also breaks down spontaneously to 3-phosphoglyceric acid and inorganic phosphate, but the rate of this reaction is slower than that of the enzymatic phosphate transfer. In order that the transfer of phosphate from 1,3-diphosphoglyceric acid to adenosine diphosphate may proceed nearly to completion, adenosine diphosphate must be present in excess or must be continuously regenerated from the adenosine triphosphate. Adenosine diphosphate may be formed from adenosine triphosphate as the result of hydrolysis by the enzyme adenosinetriphosphatase or as the result of a transfer of one phosphate group from adenosine triphosphate to a phosphate acceptor such as glucose (Equation 1). Magnesium ions are required as a cofactor for these phosphate transfer reactions. On the basis of these facts, one suitable system for studying the oxidation of 3-phosphoglyceraldehyde would contain stoichiometric amounts of the substrate, inorganic phosphate, glucose, and pyruvate, and catalytic amounts of DPN, adenosine diphosphate (or adenosine triphosphate), and magnesium ions. The over-all reaction occurring in this system (the sum of Equations 4, 5, 6, and 1) is represented by Equation 7. It was

$$\text{3-Phosphoglyceraldehyde} + \text{phosphate} + \text{glucose} + \text{pyruvate} \rightarrow \text{3-phosphoglycerate} + \text{H}^+ + \text{glucose-6-phosphate} + \text{lactate} \qquad (7)$$

felt that the demonstration of such an oxidation in parasite material would be strong evidence for the occurrence of a phosphorylating glycolysis in the malaria parasite.

Aldolase Reaction—Extracts of parasite material were able to split fructose-1,6-diphosphate to triose phosphate. The products of the splitting of fructose-1,6-diphosphate by the enzyme aldolase, 3-phosphoglyceraldehyde and dihydroxyacetone phosphate, may be identified by the fact that their phosphate groups are readily hydrolyzed by N NaOH at room temperature (16). Since the reaction reaches an equilibrium unless the products are removed, cyanide was added to bind the triose phosphate. In a system containing fructose-1,6-diphosphate, cyanide, and an extract of parasite material, the formation of compounds containing alkali-labile phosphate groups was observed. This indicates that the fructose-1,6-diphosphate was being split in the manner represented by Equation 3.

Coupled Oxidation-Reduction Reaction—The oxidation of 3-phosphoglyceraldehyde according to Equation 7 results in the formation of acid. Therefore the process can be followed conveniently by measuring the carbon dioxide liberated from a bicarbonate buffer containing the test system. The test system used in studying the oxidation contained magnesium, fluoride, inorganic phosphate, glucose, fructose-1,6-diphosphate, pyruvate, DPN, adenosine triphosphate, and an extract of parasite material. Fluoride was added to inhibit partially the adenosinetriphosphatase present in the extracts and to prevent the breakdown of the 3-phosphoglycerate to pyruvate (20, 21). Fructose-1,6-diphosphate was used as the substrate, since the extracts contained the enzyme aldolase.

Table II

Components of System Oxidizing 3-Phosphoglyceraldehyde

The complete system contained 0.026 M $NaHCO_3$, 0.004 M $MgSO_4$, 0.008 M phosphate at pH 7.4, 0.02 M KF, 0.013 M glucose, 0.013 M sodium pyruvate, 0.0007 M adenosine triphosphate, 0.00007 M diphosphopyridine nucleotide, 0.0035 M fructose-1,6-diphosphate (tipped in from the side arm), and 1.7 cc. of an extract of parasite material (prepared by saponization, 0.9 per cent NaCl without glucose used for all washings), in a total volume of 2.5 cc. Warburg manometers; gas phase, 5 per cent CO_2-95 per cent N_2; temperature 39°.

Sample	Rate
	microliters CO_2 per hr.
Complete system	56
Without fructose-1,6-diphosphate	44
" glucose	42
" adenosine triphosphate	41
" phosphate	39
" $MgSO_4$	34
" diphosphopyridine nucleotide	33
" pyruvate	27

Oxidation of 3-phosphoglyceraldehyde by extracts of parasite material required all the components of this test system, as shown by the data in Table II. Omission of fructose-1,6-diphosphate, glucose, adenosine triphosphate, inorganic phosphate, magnesium, DPN, or pyruvate decreased the rate of the reaction by 20 to 50 per cent. The activity of the system in this experiment was much smaller than that usually obtained, because in the preparation of the extract it was necessary to omit glucose from the wash solutions.

Effects of Arsenate and Iodoacetate—Arsenate and iodoacetate have characteristic effects on the oxidation of 3-phosphoglyceraldehyde. Arsenate is believed to replace inorganic phosphate in Reaction 4, forming

1-arseno-3-phosphoglyceric acid as the product of the oxidation; this substance breaks down rapidly and spontaneously to arsenate and 3-phosphoglyceric acid (18). Therefore, in the presence of arsenate the oxidation of 3-phosphoglyceraldehyde occurs according to Equation 8. The coupling

$$\text{3-Phosphoglyceraldehyde} + DPN^+ \rightarrow \text{3-phosphoglycerate} + DPNH + 2\,H^+ \quad (8)$$

of the oxidation with the uptake and transfer of phosphate is avoided.

TABLE III

Effect of Arsenate and Iodoacetate on Oxidation of 3-Phosphoglyceraldehyde

The complete system contained 0.026 M $NaHCO_3$, 0.004 M $MgSO_4$, 0.02 M KF, 0.0035 M fructose-1,6-diphosphate, 0.013 M sodium pyruvate, 0.00007 M diphosphopyridine nucleotide, 0.008 M phosphate at pH 7.4, 0.013 M glucose, 0.0007 M adenosine triphosphate, and 1.7 cc. of extract of parasite material, in a total volume of 2.5 cc. The arsenate sample contained 0.0012 M Na_2HAsO_4 and no phosphate, glucose, or adenosine triphosphate. The iodoacetate sample was like the arsenate sample with the addition of 0.0012 M sodium iodoacetate. Warburg manometers; gas phase, 5 per cent CO_2-95 per cent N_2; temperature 39°.

Sample	Rate
	microliters CO_2 per hr.
Complete	143
Arsenate	221
Iodoacetate	19

TABLE IV

Lactic Dehydrogenase Activity of Parasite Extracts

The complete system contained 0.02 M phosphate at pH 7.4, 54 γ of sodium dichlorophenol indophenol, 0.1 M lithium *dl*-lactate, 0.00003 M diphosphopyridine nucleotide, and 0.4 cc. of extract, in a total volume of 5.0 cc. The parasite material was prepared by hemolyzing the red cells, and the extract was dialyzed against 0.9 per cent NaCl for 4 hours at 0°. Temperature 21°. ΔG represents the increase in percentage transmission, measured in an Evelyn photoelectric colorimeter with Filter 620.

Sample	Rate, ΔG in 4 min.
Complete	10.2
Without lactate	4.0
" diphosphopyridine nucleotide	4.2

With arsenate present instead of inorganic phosphate, Reaction 7 would be changed to Reaction 9. Since the phosphate transfer reactions are

$$\text{3-Phosphoglyceraldehyde} + \text{pyruvate} \rightarrow \text{3-phosphoglycerate} + H^+ + \text{lactate} \quad (9)$$

usually slower than the oxidation itself, the addition of arsenate characteristically increases the rate of the oxidation of 3-phosphoglyceraldehyde and makes the reaction independent of the presence of inorganic phosphate,

magnesium, adenosine triphosphate, and glucose or other phosphate acceptors. The specific action of iodoacetate consists in inhibiting 3-phosphoglyceraldehyde dehydrogenase, the enzyme which catalyzes Reactions 4 and 8 (22). Few other known enzymes are equally sensitive to low concentrations (0.001 M) of iodoacetate.

Table III gives the results of an experiment on the effects of arsenate and iodoacetate on the oxidation of 3-phosphoglyceraldehyde in extracts of parasite material. Arsenate markedly accelerated the reaction, and iodoacetate caused an inhibition of more than 90 per cent.

Lactic Dehydrogenase—The presence in extracts of parasite material of the enzyme lactic dehydrogenase, which catalyzes Reaction 5, was demonstrated by means of the colorimetric technique described by Haas (23). An Evelyn photoelectric colorimeter with Filter 620 was used to measure the rate of reduction of the dye. The change in percentage transmission (ΔG) per unit time was proportional to the enzyme activity, and the rate was constant over short periods of time. The results of an experiment on the lactic dehydrogenase activity of an extract of parasite material are given in Table IV. It is difficult to obtain preparations with no activity in the absence of added DPN or lactate.

The experiments which have just been described indicate that the malaria parasite is capable of converting fructose-1,6-diphosphate to 3-phosphoglyceraldehyde and of oxidizing the latter substance by a mechanism similar to that found in muscle extracts.

DISCUSSION

No previous study has been made of the mechanism of the formation of lactic acid from glucose by protozoa. Therefore it is of interest to find that the malaria parasite glycolyzes by a process similar to that found in a wide variety of other organisms (plants, bacteria, yeast, vertebrates). In this respect, as in the oxidative reactions thus far investigated, the metabolism of the parasite conforms to a pattern common among living cells.

SUMMARY

The preparation of cell-free enzyme extracts from the malaria parasite is described. These extracts convert glucose to lactic acid by a path similar to the phosphorylating glycolysis of yeast and vertebrate muscle. The preparations contain enzymes which catalyze the phosphorylation of glucose by adenosine triphosphate, the splitting of fructose-1,6-diphosphate to form 3-phosphoglyceraldehyde, and the dismutation between 3-phosphoglyceraldehyde and pyruvic acid.

BIBLIOGRAPHY

1. Silverman, M., Ceithaml, J., Taliaferro, L. G., and Evans, E. A., Jr., *J. Infect. Dis.*, **75**, 212 (1944).
2. Wendel, W. B., *J. Biol. Chem.*, **148**, 21 (1943).
3. Gomori, G., *J. Lab. and Clin. Med.*, **27**, 955 (1942).
4. Needham, D. M., *Biochem. J.*, **36**, 113 (1942).
5. Neuberg, C., Lustig, H., and Rothenberg, M. A., *Arch. Biochem.*, **3**, 33 (1943).
6. Williamson, S., and Green, D. E., *J. Biol. Chem.*, **135**, 345 (1940).
7. Meyerhof, O., *Biochem. Z.*, **246**, 249 (1932).
8. Krebs, H. A., and Eggleston, L. V., *Biochem. J.*, **34**, 442 (1940).
9. Christophers, S. F., and Fulton, J. D., *Ann. Trop. Med. and Parasitol.*, **33**, 161 (1939).
10. Utter, M. F., and Werkman, C. H., *Biochem. J.*, **36**, 485 (1942).
11. Colowick, S. P., and Kalckar, H. M., *J. Biol. Chem.*, **148**, 117 (1943).
12. Lohmann, K., *Biochem. Z.*, **262**, 137 (1933).
13. Ostern, P., Guthke, J. A., and Terszakovec, J., *Z. physiol. Chem.*, **243**, 9 (1936).
14. Lohmann, K., *Biochem. Z.*, **202**, 466 (1928).
15. Roe, J. H., *J. Biol. Chem.*, **107**, 15 (1934).
16. Meyerhof, O., and Lohmann, K., *Biochem. Z.*, **271**, 89 (1934).
17. Meyerhof, O., *Bull. Soc. chim. biol.*, **20**, 1945 (1938).
18. Warburg, O., and Christian, W., *Biochem. Z.*, **303**, 40 (1939).
19. Bücher, T., *Naturwissenschaften*, **30**, 756 (1942).
20. Lohmann, K., and Meyerhof, O., *Biochem. Z.*, **273**, 61 (1934).
21. Warburg, O., and Christian, W., *Biochem. Z.*, **310**, 384 (1942).
22. Adler, E., von Euler, H., and Gunther, G., *Skand. Arch. Physiol.*, **80**, 1 (1938).
23. Haas, E., *J. Biol. Chem.*, **155**, 333 (1944).

THE BIOCHEMISTRY OF THE MALARIA PARASITE*

III. THE EFFECTS OF QUININE AND ATABRINE ON GLYCOLYSIS

By JOHN F. SPECK and E. A. EVANS, Jr.

(*From the Department of Biochemistry of the University of Chicago, Chicago*)

(Received for publication, February 15, 1945)

In Paper II (1) it was shown that the formation of lactic acid from glucose by the malaria parasite is a process similar to the phosphorylating glycolysis of yeast and vertebrate muscle. Quinine and atabrine have been found to inhibit the glycolysis (2) as well as the oxygen uptake (2–4) of the parasite. Since glycolysis is a preliminary stage in the oxidation of glucose under aerobic conditions and is the only path for the utilization of glucose anaerobically, it was believed that an investigation of the effects of antimalarial drugs on this process might aid in understanding the mode of action of these drugs.

The present paper describes the action of quinine and atabrine on the phosphorylation of glucose and the dehydrogenation of 3-phosphoglyceraldehyde and lactic acid by preparations of the avian parasite *Plasmodium gallinaceum*. Comparable experiments with glycolytic enzymes from yeast and mammalian muscle are also reported.

Methods and Materials

Inorganic phosphate was determined by the method of Gomori (5) on trichloroacetic acid filtrates. Quinine interferes with the estimation of phosphate by the molybdenum blue method by forming a precipitate with phosphomolybdic acid, and atabrine interferes because of its deep yellow color. Both difficulties can be avoided by extracting the free bases from alkaline solution with an immiscible solvent. The solution to be analyzed for phosphate is made alkaline to phenolphthalein, extracted twice with an equal volume of carbon tetrachloride, and clarified by centrifugation.

Solutions of quinine and atabrine for use in the enzyme experiments were prepared by neutralizing solutions of the dihydrochlorides with 1.5 equivalents of sodium bicarbonate. The final concentration of the neutralized solutions was not made greater than 0.01 M; otherwise the bases did not remain in solution long enough to permit their being pipetted into the reaction mixtures.

* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Chicago. This work was also supported in part by grants from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

Adenosine triphosphate, fructose-1,6-diphosphate, and diphosphopyridine nucleotide were prepared as described in Paper II (1). The glycogen and adenylic acid were commercial preparations. Potassium glucose-1-phosphate was obtained essentially by the method of Hanes (6). In our experience, after removal of barium with sulfuric acid and neutralization with potassium hydroxide, the potassium glucose-1-phosphate did not precipitate on addition of an equal volume of alcohol. It could be obtained in crystalline form by adding more alcohol to the supernatant and allowing it to stand in the cold. Phosphoglyceric acid was prepared according to Neuberg and Kobel (7). Phosphopyruvic acid was synthesized by a modification of the method of Kiessling (8) suggested by Dr. Gerhardt Schmidt.[1] Adenosine diphosphate was prepared from adenosine triphosphate by the use of yeast hexokinase (9).

The preparation of enzyme extracts from malaria parasites (*Plasmodium gallinaceum*) is described in Paper II (1). The enzymes from yeast and muscle are described in connection with the particular reaction for which they were used.

Results

Effects of Quinine and Atabrine on Glycolysis in Preparations from Malaria Parasites

Phosphorylation of Glucose—The effect of quinine and atabrine on the phosphorylation of glucose by hemolysates of normal and parasitized chicken red blood cells was studied with the manometric system described in Paper II (1). The results of several experiments are summarized in Table I. Quinine in concentrations from 0.0001 to 0.002 M inhibited the phosphorylation of glucose in hemolysates of normal chicken red cells but caused only a slight inhibition in hemolysates of parasitized red cells. The inhibition in the latter case was not greater than would be expected from inhibition of that fraction of the total hexokinase activity (about one-fourth) derived from the enzymes of the red cells themselves. However, atabrine in the same concentrations as quinine caused an equal inhibition of the phosphorylation of glucose, in hemolysates of both normal and parasitized erythrocytes.

3-Phosphoglyceraldehyde Dehydrogenase—The effects of quinine and atabrine on the enzyme 3-phosphoglyceraldehyde dehydrogenase were studied in a system in which the over-all process was that described by Equation 1. Three enzymatic reactions are involved in this process:

$$\text{Fructose-1,6-diphosphate} + \text{pyruvate} \rightarrow \text{3-phosphoglycerate} + H^{+} + \text{lactate} + \text{dihydroxyacetone phosphate} \quad (1)$$

(1) the splitting of fructose-1,6-diphosphate to 3-phosphoglyceraldehyde

[1] Schmidt, G., personal communication.

and dihydroxyacetone phosphate (catalyzed by the enzyme aldolase); (2) the oxidation of 3-phosphoglyceraldehyde by diphosphopyridine nucleotide in the presence of arsenate to form 3-phosphoglyceric acid and reduced diphosphopyridine nucleotide (catalyzed by the enzyme 3-phosphoglyceraldehyde dehydrogenase); and (3) the reoxidation of the reduced diphosphopyridine nucleotide by pyruvate to form diphosphopyridine nucleotide and lactate (catalyzed by the enzyme lactic dehydrogenase) (see the discussion of the oxidation-reduction reactions in Paper II (1)). An excess of purified preparations of the enzymes aldolase and lactic dehydrogenase was added; therefore the rate of the over-all process was determined by the activity of the 3-phosphoglyceraldehyde dehydrogenase.

TABLE I

Effect of Quinine and Atabrine on Hexokinase Activity of Red Blood Cell Hemolysates

The sample contained 0.028 M $NaHCO_3$, 0.004 M $MgSO_4$, 0.028 M KF, 0.012 M glucose, 0.0036 M adenosine triphosphate (added from the side arm after equilibration), 1.4 cc. of hemolysate of normal chicken red cells or 0.7 cc. of hemolysate of parasitized red cells, and the concentrations of quinine and atabrine indicated below, in a total volume of 2.5 cc. Warburg manometers; gas phase, 5 per cent CO_2-95 per cent N_2; temperature 39°.

Concentration of inhibitor	Per cent inhibition by			
	Quinine		Atabrine	
	Normal	Parasitized	Normal	Parasitized
M				
0.0001	6	4	6	8
0.0005	18	4	8	18
0.001	20	8	31	26
0.002	43	10	40	39

Aldolase was purified according to Herbert *et al.* (10) through the stage of the first fractionation with ammoniacal ammonium sulfate. Lactic dehydrogenase was prepared from beef heart by following the method of Straub (11) through the second ammonium sulfate precipitation. Since acid is formed, the reaction was followed by measuring the evolution of carbon dioxide from a bicarbonate buffer containing the test system. The complete test system contained bicarbonate, arsenate, fluoride, fructose-1,6-diphosphate, pyruvate, and the three enzymes aldolase, 3-phosphoglyceraldehyde dehydrogenase, and lactic dehydrogenase. If only aldolase and lactic dehydrogenase were added, no acid was formed. Therefore these two preparations were free from 3-phosphoglyceraldehyde dehydrogenase activity. When a preparation containing 3-phosphoglyceraldehyde dehydrogenase was added, in addition to the other two enzymes, acid was formed at a rate which was proportional to the amount of dehydrogenase

added and was constant until at least one-third of the fructose-1,6-diphosphate had been used. Extracts of parasite material (1) as well as preparations made from rabbit skeletal muscle were effective as sources of 3-phosphoglyceraldehyde dehydrogenase.

The effect of quinine and atabrine on the enzyme 3-phosphoglyceraldehyde dehydrogenase extracted from parasite material was investigated by the use of the system just described. In concentrations up to 0.002 M neither drug inhibited this enzyme significantly. These results are shown in Table II.

Lactic Dehydrogenase—The effect of quinine and atabrine on the enzyme lactic dehydrogenase in extracts of parasite material was studied by means

TABLE II

Effect of Quinine and Atabrine on 3-Phosphoglyceraldehyde Dehydrogenase in Parasite Extracts

The samples contained 0.026 M $NaHCO_3$, 0.005 M Na_2HAsO_4, 0.03 M NaF, 0.01 M sodium pyruvate, 0.005 M fructose-1,6-diphosphate (added from side arm after equilibration), 0.00037 M diphosphopyridine nucleotide, aldolase preparation containing 1 mg. of protein, lactic dehydrogenase preparation containing 3 mg. of protein, 0.8 cc. of an extract of parasite material (prepared by hemolysis), and the concentrations of quinine and atabrine indicated below, in a total volume of 2.5 cc. Warburg manometers; gas phase, 5 per cent CO_2-95 per cent N_2; temperature 39°.

Concentration of inhibitor	Activity of dehydrogenase	
	Quinine	Atabrine
M	*microliters CO_2 per 5 min.*	*microliters CO_2 per 5 min.*
0	14.6	12.6
0.0001	14.0	12.8
0.0003	14.0	13.2
0.001	14.1	12.5
0.002	14.2	12.9

of the colorimetric technique of Haas (12), as described in Paper II (1). The results of two experiments are shown in Table III. Both drugs inhibited the enzyme, but atabrine was considerably more effective than quinine.

Effect of Quinine and Atabrine on Yeast Hexokinase

The enzyme hexokinase in yeast catalyzes the transfer of one phosphate group from adenosine triphosphate to glucose, to form glucose-6-phosphate and adenosine diphosphate (9). This same reaction is apparently the first step in the phosphorylation of glucose by the malaria parasite also (1).

Hexokinase was prepared from bakers' yeast according to Colowick and Kalckar (9), and the reaction was studied manometrically in a test system

similar to theirs. Determination of the labile pyrophosphate groups of adenosine triphosphate (phosphate hydrolyzed by 1 N H_2SO_4 in 10 minutes at 100° (13)) showed that the formation of acid was accompanied by a decrease in pyrophosphate groups and the formation of a more stable phosphate compound.

TABLE III

Effect of Quinine and Atabrine on Lactic Dehydrogenase in Parasite Extracts

The samples contained 0.02 M phosphate at pH 7.4, 54 γ of sodium dichlorophenol indophenol, 0.1 M lithium *dl*-lactate, 0.00003 M diphosphopyridine nucleotide, 0.4 cc. of an extract of parasite material, and the concentrations of quinine and atabrine indicated below, in a total volume of 5.0 cc.; temperature 20°. The rate of reaction was measured as the increase in percentage transmission per unit time, read in an Evelyn photoelectric colorimeter with Filter 620.

Concentration of inhibitor	Per cent inhibition by	
	Quinine	Atabrine
M		
0.0001	5	14
0.0003	7	26
0.001	16	38

TABLE IV

Effect of Quinine and Atabrine on Yeast Hexokinase

The samples contained 0.02 M $NaHCO_3$, 0.01 M $MgSO_4$, 0.02 M glucose, 0.005 M adenosine triphosphate (tipped in from the side arm after equilibration), hexokinase preparation containing 1.0 mg. of protein, and the concentrations of quinine and atabrine indicated below, in a total volume of 2.0 cc. Warburg manometers; gas phase, 5 per cent CO_2-95 per cent N_2; temperature 30°. The rate of reaction was about 140 microliters of CO_2 per hour.

Concentration of inhibitor	Per cent inhibition by	
	Quinine	Atabrine
M		
0.0005	6	2
0.001	16	8
0.002	23	22

The average results of seven experiments with quinine and the results of a single experiment with atabrine are given in Table IV. Both quinine and atabrine are seen to inhibit the action of yeast hexokinase.

Effect of Quinine on Glycolysis by Muscle Enzymes

Acid Formation from Glycogen—The over-all reaction of glycolysis in rabbit muscle extracts is the formation of lactic acid from glycogen. Since

acid is produced, the course of the reaction can be followed by measuring the evolution of carbon dioxide from a bicarbonate buffer containing the test system (14). The system used contained 0.028 M $NaHCO_3$, 0.007 M phosphate at pH 7.4, 9 mg. of glycogen, and 50 mg. of powder obtained by lyophilizing an aqueous extract of rabbit skeletal muscle (14), in a volume of 3.0 cc. The samples were placed in Warburg manometers under an atmosphere of 5 per cent CO_2-95 per cent N_2, at a temperature of 35°, and the glycogen was tipped in from the side arm after equilibration. About 300 microliters of CO_2 per hour were evolved.

Quinine consistently inhibited the formation of acid in this system. The average results from several experiments indicated an inhibition of 8 per cent at a quinine concentration of 0.0005 M, of 14 per cent at 0.001 M, and of 31 per cent at 0.002 M. The inhibition was greatest when the enzyme was incubated with quinine before the reaction was started. When the quinine was tipped in at the same time as the glycogen, smaller inhibitions were observed.

Phosphorylase—The phosphorylation of glycogen in muscle extracts occurs in the manner indicated in Equation 2 (15). Phosphorylase, the

$$\underset{\text{Glycogen}}{(C_6H_{10}O_5)_n} + n\mathrm{HPO_4^{=}} \rightleftharpoons \underset{\text{Glucose-1-phosphate}}{nC_6H_{11}O_6PO_3^{=}} \qquad (2)$$

enzyme catalyzing this reaction, was prepared from rabbit muscle extract according to Green, Cori, and Cori (16). It was not crystalline but was active and free from phosphoglucomutase activity (conversion of glucose-1-phosphate to glucose-6-phosphate).

The forward reaction, formation of glucose-1-phosphate, was studied in a system containing the enzyme phosphorylase, inorganic phosphate, glycogen, adenylic acid (as coenzyme), and reduced glutathione (to insure maximum activity of the enzyme). The reaction was followed by determining the decrease in inorganic phosphate. The results of experiments on the effect of quinine on the phosphorylation of glycogen are given in Table V. Quinine markedly inhibited this reaction. The inhibition was proportional to the concentration of quinine and decreased with time.

The reverse reaction, formation of polysaccharide from glucose-1-phosphate, was studied in a system like that of Cori and Cori (17). The process was followed by measuring the increase in inorganic phosphate. A marked inhibition by quinine was observed, just as with the forward reaction. The degree of inhibition was proportional to the concentration of quinine and decreased with time. These results are illustrated in Table VI.

Since these experiments measured the rate at which the reactions approached an equilibrium, a decrease in inhibition with longer periods of time was to be expected.

Phosphoglucomutase—The enzyme phosphoglucomutase catalyzes the attainment of an equilibrium between glucose-1-phosphate and glucose-6-phosphate (18). The enzyme used in these experiments was prepared according to Colowick and Sutherland (19), and the test system was similar to theirs.

The results of a typical experiment on the effect of quinine on phosphoglucomutase are shown in Table VII. Quinine inhibited the conversion

TABLE V

Effect of Quinine on Phosphorolysis of Glycogen

The samples contained 0.02 M reduced glutathione, 0.001 M adenylic acid, 0.05 M phosphate at pH 7.2, 1 per cent glycogen, phosphorylase preparation containing 6 mg. of protein, and the concentrations of quinine indicated below, in a total volume of 4 cc.; temperature 25°.

Time	Decrease in inorganic P		Per cent inhibition		
			Concentration of quinine		
	No quinine	0.001 M quinine	0.0005 M	0.001 M	0.002 M
min.	*γ per cc.*	*γ per cc.*			
20	180	90	37	50	100
40	250	190	19	24	58

TABLE VI

Effect of Quinine on Polysaccharide Formation from Glucose-1-Phosphate

The samples contained 0.05 M glycerophosphate at pH 6.65, 0.02 M reduced glutathione, 0.001 M adenylic acid, 0.5 per cent glycogen, 0.016 M potassium glucose-1-phosphate, phosphorylase preparation containing 4 mg. of protein, and the concentrations of quinine indicated below, in a total volume of 4.0 cc.; temperature 20°.

Time	Increase in inorganic P		Per cent inhibition		
			Concentration of quinine		
	No quinine	0.001 M quinine	0.0005 M	0.001 M	0.002 M
min.	*γ per cc.*	*γ per cc.*			
5	132	76	17	42	76
10	252	161	14	36	60

of glucose-1-phosphate to glucose-6-phosphate, but to a variable extent.

Phosphorylation of Fructose-6-phosphate—Fructose-6-phosphate is converted to fructose-1,6-diphosphate by a transfer of phosphate from adenosine triphosphate, catalyzed by the enzyme phosphohexokinase (20). Since this transfer results in the liberation of acid, rate studies can conveniently be made by measuring the evolution of carbon dioxide when the reaction takes place in a bicarbonate buffer. The reaction mixture used

in these experiments contained 0.02 M $NaHCO_3$, 0.006 M $MgSO_4$, 0.0012 M sodium iodoacetate, 0.012 M potassium glucose-1-phosphate, 0.004 M adenosine triphosphate (tipped in from the side arm after equilibration), and a dialyzed extract equivalent to 80 mg. of an acetone powder of rabbit muscle (21), in a total volume of 2.5 cc. The gas phase in the Warburg manometers was 5 per cent CO_2-95 per cent N_2, and the temperature was 37°. The iodoacetate prevented liberation of acid by further reaction of the fructose-1,6-diphosphate formed. Glucose-1-phosphate was used as the substrate instead of fructose-6-phosphate because of its availability. During the preliminary incubation before the adenosine triphosphate was tipped in and during the subsequent experimental period, the glucose-1-phosphate was converted to glucose-6-phosphate and the latter substance rearranged to fructose-6-phosphate by the enzymes phosphoglucomutase and phosphohexoisomerase, which were present in the acetone powder

TABLE VII

Effect of Quinine on Phosphoglucomutase

The samples contained 0.04 M veronal buffer at pH 7.4, 0.005 M $MgSO_4$, 0.5 per cent reduced glutathione, 0.0065 M potassium glucose-1-phosphate, enzyme solution containing 12.5 γ of protein, and the concentrations of quinine indicated below, in a total volume of 1.0 cc. Incubated for 5 minutes at 30°.

Quinine concentration	Decrease in glucose-1-phosphate P	Inhibition
M	*γ per cc.*	*per cent*
0	127	
0.0005	108	15
0.001	109	14
0.002	73	42

extract. Since the phosphate groups of fructose-6-phosphate and fructose-1,6-diphosphate are less easily hydrolyzable than the pyrophosphate groups of adenosine diphosphate and triphosphate, the reaction can be followed approximately by measuring the decrease in pyrophosphate (phosphate liberated by hydrolysis with 1 N H_2SO_4 for 10 minutes at 100° (13)). Experiments in which organic phosphate fractions were followed showed that acid production was accompanied by a decrease in pyrophosphate groups. Control manometric experiments demonstrated that no acid was formed from adenosine triphosphate in the absence of glucose-1-phosphate, from glucose-1-phosphate in the absence of adenosine triphosphate, or from fructose-1,6-diphosphate. The rate of reaction in the complete system was about 160 microliters of CO_2 in 30 minutes.

Quinine in concentrations from 0.0005 to 0.002 M caused no inhibition of the phosphorylation of fructose-6-phosphate.

Aldolase—The enzyme aldolase catalyzes the splitting of fructose-1,6-

diphosphate to 3-phosphoglyceraldehyde and dihydroxyacetone phosphate. This enzyme was purified according to Herbert *et al.* (10) through the stage of heat coagulation, and the reaction was studied in a test system similar to the one described by these authors. Since the reaction reaches an equilibrium unless the products are removed, cyanide was added to bind the triose phosphate. Samples contained 0.05 M borate buffer at pH 7.3, 0.1 M HCN at pH 7.3, 0.012 M fructose-1,6-diphosphate, and 0.3 cc. of enzyme solution, in a total volume of 4.0 cc. These samples were incubated at 37°. Since the phosphate groups of both molecules of triose phosphate are hydrolyzed by treatment with 1 N NaOH for 20 minutes at room temperature while those of fructose-1,6-diphosphate are not

TABLE VIII

Effect of Quinine and Atabrine on 3-Phosphoglyceraldehyde Dehydrogenase from Rabbit Muscle

The samples contained 0.03 M $NaHCO_3$, 0.005 M Na_2HAsO_4, 0.03 M NaF, 0.02 M sodium pyruvate, 0.00037 M diphosphopyridine nucleotide, 0.005 M fructose-1,6-diphosphate (tipped in from the side arm after equilibration), aldolase preparation containing 1 mg. of protein, lactic dehydrogenase preparation containing 4 mg. of protein, dialyzed extract equivalent to 5 mg. of acetone powder of rabbit muscle extract (21), and the concentrations of quinine and atabrine indicated below, in a total volume of 2.5 cc. Warburg manometers; gas phase, 5 per cent CO_2-95 per cent N_2; temperature 30°.

Concentration of inhibitor	Activity of dehydrogenase	
	Quinine	Atabrine
M	*microliters CO_2 per 5 min.*	*microliters CO_2 per 5 min.*
0	10.9	13.7
0.0001	10.7	13.1
0.0003	9.9	12.8
0.001	10.0	12.6
0.002	10.4	12.0

affected, the reaction was followed by determining the increase in alkali-labile phosphate (22). In 5 minutes about 220 γ of alkali-labile P per cc. were formed.

Quinine in concentrations from 0.0005 to 0.002 M caused no significant inhibition of the enzyme aldolase.

3-Phosphoglyceraldehyde Dehydrogenase—The effect of quinine and atabrine on the enzyme 3-phosphoglyceraldehyde dehydrogenase from rabbit muscle was investigated by means of the test system involving Reaction 1, which was used to study the activity of the same enzyme in parasite extracts. As in the latter case neither quinine nor atabrine caused a significant inhibition of 3-phosphoglyceraldehyde dehydrogenase. The results of two experiments are given in Table VIII.

Lactic Dehydrogenase—The action of quinine and atabrine on the enzyme lactic dehydrogenase obtained from beef heart was studied with the aid of the colorimetric technique of Haas (12), as described in Paper II (1). The enzyme was purified by the method of Straub (11) through the stage of the second ammonium sulfate precipitation. In the test system the enzyme showed no activity in the absence of lactate or diphosphopyridine nucleotide.

Lactic dehydrogenase from beef heart was strongly inhibited by atabrine but was not significantly affected by quinine. The experimental data are given in Table IX. The results are similar to those obtained with lactic dehydrogenase from parasite extracts.

TABLE IX

Effect of Quinine and Atabrine on Lactic Dehydrogenase from Beef Heart

The samples contained 0.03 M phosphate buffer at pH 7.2, 54 γ of sodium dichlorophenol indophenol, 0.1 M lithium *dl*-lactate, 0.00003 M diphosphopyridine nucleotide, lactic dehydrogenase preparation containing 1.1 mg. of protein, and the concentrations of quinine and atabrine indicated below, in a total volume of 5.0 cc.; temperature 25°. The rate of reaction was measured as the increase in percentage transmission (ΔG) per unit time, read in an Evelyn photoelectric colorimeter with Filter 620. Control samples showed a value for ΔG of 16.0 in 4 minutes.

Concentration of inhibitor	Per cent inhibition by	
	Quinine	Atabrine
M		
0.0001	2	9
0.0003	0	16
0.001	6	41

Rearrangement of 3-Phosphoglycerate to Phosphopyruvate—The first steps in the breakdown of 3-phosphoglycerate in muscle extracts are rearrangement to 2-phosphoglycerate and dehydration of the latter substance to phosphopyruvate (23, 24) (Equation 3). The conversion of 3-phosphoglyc-

$$\begin{array}{l} COO^- \\ | \\ CHOH \\ | \\ CH_2OPO_3^= \end{array} \rightleftharpoons \begin{array}{l} COO^- \\ | \\ CHOPO_3^= \\ | \\ CH_2OH \end{array} \rightleftharpoons \begin{array}{l} COO^- \\ | \\ COPO_3^= + H_2O \\ \| \\ CH_2 \end{array} \qquad (3)$$

erate to phosphopyruvate can be followed by determining the increase in phosphopyruvate phosphate, which is released on oxidation of the phosphopyruvate by hypoiodite (23). The reaction mixture used in studying this process contained 0.05 M veronal buffer at pH 8.2, 0.005 M $MgSO_4$, 0.016 M 3-phosphoglycerate, and dialyzed extract equivalent to 4 or 5 mg. of

acetone powder of rabbit muscle (21), in a total volume of 1.0 cc. The samples were incubated for 10 minutes at 37°. Control samples formed about 125 γ of phosphopyruvate P per cc.

Quinine in concentrations from 0.0002 to 0.002 M did not inhibit the conversion of 3-phosphoglycerate to phosphopyruvate.

Phosphate Transfer from Phosphopyruvate to Adenosine Diphosphate—Phosphopyruvate is broken down during glycolysis by transfer of its phosphate group to adenylic acid or adenosine diphosphate, to form pyruvate and adenosine triphosphate (25, 26) (Equation 4). This reaction must be studied in the absence of the enzyme adenosinetriphosphatase,

$$\text{Phosphopyruvate} + \text{adenosine diphosphate} + H_2O \rightarrow \text{pyruvate} + \text{adenosine triphosphate} + OH^- \quad (4)$$

which would hydrolyze the adenosine triphosphate formed. 3-Phosphoglycerate must also be absent; otherwise the adenosine triphosphate would break down by transfer of its phosphate to 3-phosphoglycerate to form 1,3-diphosphoglyceric acid, which spontaneously decomposes to 3-phosphoglycerate and inorganic phosphate (27). An acetone powder of an aqueous rabbit muscle extract (21) was used as the source of the enzyme in studying the transfer of phosphate from phosphopyruvate to adenosine diphosphate; such a powder does not contain an active adenosinetriphosphatase. Fluoride was added to prevent the formation of 3-phosphoglycerate from the phosphopyruvate (23, 28). Samples contained 0.025 M veronal buffer at pH 8.2, 0.005 M $MgSO_4$, 0.06 M KCl (29), 0.01 M NaF, 0.005 M phosphopyruvate, 0.0045 M adenosine diphosphate, and dialyzed extract equivalent to 10 mg. of acetone powder, in a total volume of 1.0 cc. In 10 minutes at 37° about 100 γ of phosphopyruvate P per cc. were transferred.

Quinine in concentrations from 0.0005 to 0.002 M caused no inhibition of this phosphate transfer.

Phosphate Transfer from 3-Phosphoglycerate to Creatine—Muscle extract

$$\text{Adenosine triphosphate} + \text{creatine} \rightleftharpoons \text{adenosine diphosphate} + \text{phosphocreatine} + H^+ \quad (5)$$

catalyzes the transfer of phosphate from adenosine triphosphate to creatine (26) (Equation 5). The effect of quinine on the over-all process of phosphate transfer from 3-phosphoglycerate to creatine (the sum of Equations 3 to 5) was investigated. The reaction mixture consisted of 0.025 M veronal buffer at pH 8.7, 0.005 M $MgSO_4$, 0.06 M KCl, 0.03 M creatine, 0.00035 M adenosine triphosphate, 0.016 M 3-phosphoglycerate, and dialyzed extract equivalent to 5 mg. of acetone powder of rabbit muscle extract (21), in a total volume of 1.0 cc. The samples were incubated for 15 minutes at 37°. The reaction was followed by determining the increase in phosphocrea-

tine phosphate by the calcium precipitation method of Fiske and Subbarow (30). Balance experiments showed that the increase in phosphocreatine and inorganic phosphate accounted for 94 per cent of the 3-phosphoglycerate phosphate which disappeared, the rest being completely accounted for in phosphopyruvate and adenosine triphosphate. During the experimental period 240 γ of phosphocreatine P and 40 γ of inorganic P per cc. of reaction mixture were formed.

Quinine in concentrations from 0.0005 to 0.002 M caused no inhibition of the transfer of phosphate from 3-phosphoglycerate to creatine.

DISCUSSION

Silverman *et al.* (2) have found that the aerobic and anaerobic glycolysis of chicken erythrocytes parasitized with *Plasmodium gallinaceum* is inhibited about 35 per cent by 0.001 M quinine; *i.e.*, by concentrations of the drug equal to those used in the studies reported in this paper. It would appear from our data that the effects described by Silverman *et al.* are in part due to the action of the drugs on the hexokinase and lactic dehydrogenase of the parasite, since these enzymes catalyze essential steps in the glycolytic process. The inhibition by quinine of the over-all process of lactic acid formation from glycogen by rabbit muscle enzymes is due to the effect of quinine on the enzyme phosphorylase. Likewise, the inhibition of the fermentation of glucose by intact yeast cells observed by Enders and Wieninger (31) may be ascribed to the inhibition of yeast hexokinase by the antimalarial.

In connection with the latter enzyme it should be pointed out that the hexokinase from parasitized red blood cells appears to be less sensitive to quinine than does the hexokinase of yeast and normal chicken erythrocytes, although the effect of atabrine on the enzymes from the three different sources is the same. We have not, as yet, any explanation for this difference in behavior. The other glycolytic enzymes from yeast and muscle show the same sensitivity to quinine and atabrine as the corresponding enzymes of the malaria parasites.

It should be emphasized that the concentrations of antimalarials needed to produce the effects described in this paper are considerably greater than those encountered in the blood and tissue fluids of animals being treated with the drugs. In recent studies on the *in vitro* distribution of quinine between parasitized chicken erythrocytes and a suspending medium, carried out by Dr. Joseph Ceithaml in this laboratory,[2] it has been found that the ratio of intracellular to extracellular quinine concentrations is not greater than 50, even under circumstances which greatly favor the accumulation of quinine in the red blood cells. The concentration of quinine

[2] Ceithaml, J. J., and Evans, E. A., Jr., unpublished work.

observed in the red cells of chickens receiving therapeutic doses of the drug is about 10^{-5} M (32). Therefore, unless there occurs a localization of the drugs inside the parasite cell, capable of producing high concentrations of the antimalarials in the immediate vicinity of the sensitive enzymes, it seems unlikely that inhibition of the glycolytic mechanism of the parasite is primarily involved in the therapeutic action of the drugs.

At present it seems more likely that quinine and atabrine inhibit oxidative processes in the metabolism of the malaria parasite, particularly those concerned with the oxidation of lactic and pyruvic acids. The oxygen consumption of the parasite utilizing glucose is inhibited by quinine in concentrations as low as 10^{-5} M (3). With these concentrations there occurs an accumulation of lactic acid, although the rate of glucose utilization is not affected (2). Therefore, it would appear that the effect of the drug is to inhibit the oxidative removal of lactic acid rather than its formation by the process of glycolysis.

SUMMARY

The effects of quinine and atabrine on some of the glycolytic enzymes of the malaria parasite (*Plasmodium gallinaceum*), yeast, and mammalian muscle were studied.

Atabrine inhibited the hexokinase activity and the enzyme lactic dehydrogenase in parasite preparations. Quinine was less effective in both cases. The enzyme 3-phosphoglyceraldehyde dehydrogenase was not affected by either drug.

Both quinine and atabrine inhibited yeast hexokinase.

Quinine inhibited the enzymes phosphorylase and phosphoglucomutase from rabbit muscle. Lactic dehydrogenase from beef heart was strongly inhibited by atabrine but only slightly affected by quinine. The other glycolytic enzymes from rabbit muscle which were studied were not affected by quinine.

The possible significance of these effects in the action of antimalarial drugs is discussed.

BIBLIOGRAPHY

1. Speck, J. F., and Evans, E. A., Jr., *J. Biol. Chem.*, **159**, 71 (1945).
2. Silverman, M., Ceithaml, J., Taliaferro, L. G., and Evans, E. A., Jr., *J. Infect. Dis.*, **75**, 212 (1944).
3. Fulton, J. D., and Christophers, S. F., *Ann. Trop. Med. and Parasitol.*, **32**, 77 (1938).
4. Coggeshall, L. T., and Maier, J., *J. Infect. Dis.*, **69**, 108 (1941).
5. Gomori, G., *J. Lab. and Clin. Med.*, **27**, 955 (1942).
6. Hanes, C. S., *Proc. Roy. Soc. London, Series B*, **129**, 174 (1940).
7. Neuberg, C., and Kobel, M., *Biochem. Z.*, **264**, 456 (1933).
8. Kiessling, W., *Ber. chem. Ges.*, **69**, 2331 (1936).
9. Colowick, S. P., and Kalckar, H. M., *J. Biol. Chem.*, **148**, 117 (1943).

10. Herbert, H., Gordon, V., Subrahmanyan, V., and Green, D. E., *Biochem. J.*, **34**, 1108 (1940).
11. Straub, F. B., *Biochem. J.*, **34**, 482 (1940).
12. Haas, E., *J. Biol. Chem.*, **155**, 333 (1944).
13. Lohmann, K., *Biochem. Z.*, **202**, 466 (1928).
14. Meyerhof, O., *Biochem. Z.*, **178**, 395 (1926).
15. Cori, C. F., Schmidt, G., and Cori, G. T., *Science*, **89**, 464 (1939).
16. Green, A. A., Cori, G. T., and Cori, C. F., *J. Biol. Chem.*, **142**, 447 (1942).
17. Cori, G. T., and Cori, C. F., *J. Biol. Chem.*, **135**, 733 (1940).
18. Sutherland, E. W., Colowick, S. P., and Cori, C. F., *J. Biol. Chem.*, **140**, 309 (1941).
19. Colowick, S. P., and Sutherland, E. W., *J. Biol. Chem.*, **144**, 423 (1942).
20. Ostern, P., Guthke, J. A., and Terszakovec, J., *Z. physiol. Chem.*, **243**, 9 (1936).
21. Meyerhof, O., and Kiessling, W., *Biochem. Z.*, **283**, 83 (1935).
22. Meyerhof, O., and Lohmann, K., *Biochem. Z.*, **271**, 89 (1934).
23. Lohmann, K., and Meyerhof, O., *Biochem. Z.*, **273**, 61 (1934).
24. Meyerhof, O., and Kiessling, W., *Biochem. Z.*, **276**, 238 (1935).
25. Parnas, J. K., Ostern, P., and Mann, T., *Biochem. Z.*, **272**, 64 (1934).
26. Lehmann, H., *Biochem. Z.*, **281**, 271 (1935).
27. Meyerhof, O., and Junowicz-Kocholaty, R., *J. Biol. Chem.*, **145**, 443 (1942).
28. Warburg, O., and Christian, W., *Biochem. Z.*, **310**, 384 (1942).
29. Boyer, P. D., Lardy, H. A., and Phillips, P. H., *J. Biol. Chem.*, **149**, 529 (1943).
30. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **81**, 629 (1929).
31. Enders, C., and Wieninger, F. M., *Biochem. Z.*, **293**, 22 (1937).
32. Oldham, F. K., Kelsey, F. E., Cantrell, W., and Geiling, E. M. K., *J. Pharmacol. and Exp. Therap.*, **82**, 349 (1944).

AN EVALUATION OF MICROMETHODS FOR PHOSPHOLIPID

By WILLIS A. GORTNER*

(*From the Department of Biochemistry and Pharmacology, School of Medicine and Dentistry, The University of Rochester, Rochester, New York*)

(Received for publication, February 24, 1945)

A number of analytical methods for phospholipid are in routine use by various workers in the field of lipid chemistry. Only limited data are available to show the extent of agreement or disagreement among the several methods, or to point out the most reliable means of comparing data obtained by one method with phospholipid values obtained by an entirely different analytical procedure. Some of the difficulties or discrepancies encountered in comparing data from two laboratories using even slightly different phospholipid determinations have been pointed out by Bloor (4) and Ellis and Maynard (5).

During the course of an investigation of the lipids of the developing pig fetus, data were collected on the phospholipid content of the tissues as measured by the three principal methods in common use, each being focused on either a different part of the phospholipid molecule, *viz.* the phosphorus and the fatty acids, or on the intact lipid. A comparison of the analytical results by the several methods is reported in the present paper.

EXPERIMENTAL

Extracts—Lipid extracts were prepared by exhaustively extracting the ground fetal tissue with hot alcohol-ether (3:1). The combined extracts were concentrated under a vacuum at 50° to a small volume. The concentrate was then exhaustively extracted with redistilled petroleum ether. Suitable aliquots of the clear petroleum ether solution were taken for the various analyses.

Lipid Phosphorus—An aliquot containing 2 to 4 mg. of phospholipid was evaporated to dryness, then digested with 3 ml. of 10 N H_2SO_4 and several drops of phosphorus-free 30 per cent hydrogen peroxide (superoxal) until water-clear. Excess peroxide was removed by adding 10 ml. of water and boiling down to the appearance of dense white fumes. The liquid was transferred to a 50 ml. volumetric flask, with 20 ml. of water, and an additional 3 ml. of 10 N H_2SO_4 were added.[1] The phosphorus was

* Present address, School of Nutrition, Cornell University, Ithaca, New York.

[1] This solution thus contained 6 ml. of 10 N acid as against 5 ml. in the flask containing the phosphorus standard. Insufficient acid, such as from loss of SO_3 fumes, gave erroneously low P values. A slight excess of acid, such as the above procedure assured, merely slowed the rate of color development. Loss of SO_3 fumes during digestion need not be avoided in this modification.

then determined by the colorimetric procedure of Kuttner and Lichtenstein (7), 20 to 30 minutes being allowed for full color development prior to reading. In the conversion factor, phospholipid = lipid phosphorus $\times$ 24.0, an "average phospholipid" of molecular weight 744 (*i.e.*, an equimolecular mixture of oleopalmityl lecithin and cephalin) is assumed.

Phospholipid Fatty Acids—The acetone-insoluble lipids were prepared by the technique described by Bloor (4).[2] The phospholipid fatty acids were then prepared and assayed by the oxidative method of Bloor (2). The oxidation factor of 3.60 was used to calculate fatty acids. Conversion to phospholipid was based on numerous observations that approximately 63 per cent of the weight of the phospholipid is recoverable as fatty acid, which confirms the findings of Bloor (3), Sinclair (8), Bierich and Lang (1), Haven (6), and others, who report recovering less than two-thirds of the weight of the phospholipid as fatty acid.

Phospholipid—The phospholipid determination of Bloor (4)[2] was used on aliquots of the petroleum ether solution of lipids. The oxidation factor of 3.0 was used.

Experimental Data—The data obtained with three different methods for determining phospholipid on thirty-four samples of the tissue lipids of the fetal pig are summarized in Table I.

DISCUSSION

Considerably better agreement among replicate analyses was obtained from lipid phosphorus determinations than from either of the two oxidative methods for phospholipid. Thus, the standard error averaged less than ± 0.8 per cent (the range was 0.0 to 2.0 per cent) of the mean value for the phosphorus method, whereas the standard error averaged ± 2.1 per cent of the mean of the phospholipid oxidation values and ± 2.2 per cent of the fatty acid oxidation values, ranging from 0.3 to 6.3 per cent in the former and from 0.9 to 4.9 for the latter procedure.

Aside from the reduced scattering of replicate analyses in the phosphorus method, there was little choice for one method over another. The average of the thirty-four mean values for phospholipid based on the phosphorus was 593 mg. per 100 gm. of tissue; the average of the means was 594 for both of the oxidative procedures. This is additional confirmation of the validity of the conversion factors used for the several methods. The data indicate that for the lipids of animal tissues phospholipid values obtained by the method of Bloor (4) will be comparable to values calculated from the phosphorus content when an average molecular weight of

[2] The petroleum ether solution was concentrated to a smaller volume than that used by Bloor prior to the addition of acetone, and the precipitated lipid was twice washed with acetone.

TABLE I

Mean Values for Phospholipid As Determined by Oxidation of Intact Molecule, Oxidation of Fatty Acids, and Determination of Lipid Phosphorus

The values (± the standard error of the mean) are expressed as mg. per 100 gm. of fresh tissue. The figures in parentheses are the number of replicate determinations.

Sample No.	Lipid phosphorus × 24.0	Oxidation of phospholipid	$\frac{\text{Phospholipid fatty acids}}{0.63}$
38x	248 ± 0.6 (3)	205 ± 2.6 (3)	265 ± 10.7 (3)
170	371 ± 2.0 (3)	350 ± 4.5 (3)	424 ± 16.0 (3)
162	413 (2)	421 ± 10.5 (4)	387 (2)
196	492 ± 9.1 (3)	495 ± 10.5 (4)	480 ± 10.1 (3)
189	540 ± 1.7 (3)	553 ± 11.9 (4)	534 ± 8.0 (3)
65	548 (2)	544 ± 4.0 (4)	550 ± 5.0 (3)
22	550 (2)	553 ± 11.7 (3)	614 ± 10.3 (3)
28	552 ± 1.0 (3)	555 ± 4.4 (4)	626 ± 11.5 (3)
200	553 ± 8.3 (3)	619 ± 6.6 (4)	619 ± 6.1 (3)
254	562 ± 11.4 (3)	608 ± 1.7 (4)	632 ± 7.5 (3)
187	564 ± 9.9 (3)	586 ± 7.7 (4)	558 ± 22.5 (3)
103	575 ± 8.6 (3)	658 ± 17.4 (3)	610 ± 13.8 (3)
118	592 (2)	533 ± 9.2 (4)	505 ± 8.2 (4)
135	601 ± 10.2 (3)	583 ± 4.2 (3)	635 ± 16.0 (3)
260	610 ± 1.0 (3)	611 ± 9.2 (4)	633 ± 6.0 (3)
95	611 ± 0.0 (3)	656 ± 11.9 (4)	616 ± 17.8 (3)
17	612 ± 2.0 (3)	671 ± 8.5 (4)	517 ± 13.9 (3)
72	614 ± 0.0 (3)	617 ± 5.7 (4)	581 ± 7.8 (3)
180	616 ± 0.0 (3)	593 ± 5.0 (3)	616 ± 12.0 (3)
230	617 ± 6.3 (3)	621 ± 14.5 (4)	622 (2)
107	627 ± 1.0 (3)	672 ± 7.6 (4)	754 ± 17.0 (3)
240	636 (2)	689 ± 43.1 (3)	627 ± 15.7 (3)
55	641 ± 0.0 (3)	593 ± 5.6 (4)	646 ± 10.4 (4)
29	643 ± 10.9 (3)	667 ± 25.7 (4)	675 ± 11.9 (3)
85	644 (2)	630 ± 7.4 (4)	598 ± 20.8 (3)
23	646 ± 10.3 (3)	657 ± 7.5 (4)	700 (2)
114	649 ± 0.0 (3)	554 ± 16.7 (3)	585 ± 12.4 (3)
34	658 (2)	630 ± 16.3 (4)	700 ± 12.5 (3)
105	662 ± 5.8 (3)	617 ± 13.9 (4)	533 (2)
38	681 ± 4.0 (3)	696 ± 9.1 (4)	629 ± 24.9 (3)
66	690 ± 0.0 (3)	686 ± 24.4 (4)	692 ± 6.0 (4)
21	714 ± 14.0 (3)	673 ± 14.5 (4)	674 ± 5.6 (3)
56	719 ± 2.0 (3)	751 ± 16.3 (4)	687 ± 34.0 (3)
39	726 ± 3.9 (3)	664 ± 50.8 (6)	661 ± 21.2 (3)

744 is assumed for the mixed phospholipids, and to values calculated from the phospholipid fatty acids when an average recovery of 63 per cent of the weight of the phospholipid is assumed.

It should be emphasized that any conversion factor is subject to error

when a mixture of lecithin, cephalin, and sphingomyelin is to be analyzed, since the relative distribution of the three fractions as well as the molecular weights of the fatty acids present affect the conversion factor. Close agreement among the three phospholipid methods will not be found when the composition of the sample varies significantly from the conditions assumed in making the calculations. Thus, it was not surprising to note that, although there was generally satisfactory agreement among the several methods, in certain instances one procedure gave results quite different from the other two. For example, there was excellent agreement between the two oxidation methods in the case of Samples 200 and 21, but the phospholipid content as determined from the phosphorus analysis was much lower in the former, and in the latter sample was considerably higher than the content as determined oxidatively. Similarly, in Samples 28 and 72 the lipid phosphorus values agreed closely with those obtained from oxidation of the intact phospholipid, while the contents indicated by the fatty acid determination differed significantly, in one case being higher and in the other, lower.

The data confirm the findings of others that although a conversion factor may prove fairly reliable in the "average" lipid sample, it may lead to erroneously low or high values for phospholipid in some instances. The unpredictability of these cases in which the factor is incorrect is the unavoidable, inherent fault of any micromethod for phospholipid. The lipid phosphorus analysis, however, will give a fairly true picture of the *molar concentration* if not of the *weight* of the phospholipid sample.

SUMMARY

Thirty-four lipid extracts of animal tissue have been analyzed for phospholipid by lipid phosphorus determination, by oxidation of the fatty acids from the acetone-insoluble lipids, and by direct oxidation of the intact phospholipid. When a phospholipid to phosphorus ratio of 24 was used, and when a fatty acid recovery of 63 per cent of the weight of the saponified phospholipid was assumed, the three methods gave comparable results. Considerably better agreement among replicate samples was obtained by phosphorus analyses than by either of the two oxidative procedures studied.

BIBLIOGRAPHY

1. Bierich, R., and Lang, A., *Z. physiol. Chem.*, **216**, 217 (1933).
2. Bloor, W. R., *J. Biol. Chem.*, **77**, 53 (1928).
3. Bloor, W. R., *J. Biol. Chem.*, **80**, 443 (1928).
4. Bloor, W. R., *J. Biol. Chem.*, **82**, 273 (1929).
5. Ellis, G., and Maynard, L. A., *J. Biol. Chem.*, **118**, 701 (1937).
6. Haven, F. L., *Am. J. Cancer*, **29**, 57 (1937).
7. Kuttner, T., and Lichtenstein, L., *J. Biol. Chem.*, **86**, 671 (1930).
8. Sinclair, R. G., Thesis, University of Rochester (1928).

TRYPTOPHANE METABOLISM

XI. CHANGES IN THE CONTENT OF TRYPTOPHANE, UREA, AND NON PROTEIN NITROGEN IN THE BLOOD AND OF TRYPTOPHANE IN THE TISSUES AFTER THE ORAL ADMINISTRATION OF TRYPTOPHANE OR TRYPTOPHANE DERIVATIVES*

BY DOROTHY M. BUCK AND CLARENCE P. BERG

(*From the Biochemical Laboratory, State University of Iowa, Iowa City*)

(Received for publication, February 26, 1945)

Most of the information available on tryptophane metabolism is concerned with growth or the excretion of metabolites. It seemed to us that an examination of the blood at intervals after the administration of tryptophane might afford further evidence particularly as to rate and course of metabolism in animals known to excrete kynurenic acid. Accordingly the blood of rabbits fed tryptophane was assayed at intervals for tryptophane, urea, and non-protein nitrogen. Ultimately similar tests were undertaken after feeding derivatives of tryptophane known to be unavailable for growth in the rat. The distribution of tryptophane in the tissues of the rat fed tryptophane was also investigated.

The studies are similar to those on other amino acids recorded some years ago by Luck (1), Johnston and Lewis (2), and Shambaugh, Lewis, and Tourtellotte (3). They differ chiefly in the use of a relatively specific test for the amino acid fed instead of a test for amino nitrogen, and in the administration of the tryptophane and tryptophane derivatives as suspensions, without neutralization or conversion to the sodium salt. The data show differences which seem to be significant and probably associable with known peculiarities in the metabolism of tryptophane.

EXPERIMENTAL

Comparisons of several methods for determining the relatively small concentrations of tryptophane in the blood after feeding tryptophane led to the adoption of the Shaw and McFarlane procedure (4) with minor modification; this is essentially a quantitative adaptation of the Hopkins-Cole glyoxylic acid test. The extinction coefficient of the colored solution was measured in a Zeiss Pulfrich photometer; Filter S-53 (530 mμ) was used and the test solution balanced against a blank prepared in the same

* The experimental data in this paper are from a dissertation submitted by Dorothy M. Buck in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biochemistry in the Graduate College of the State University of Iowa.

way, but without the glyoxylic acid. The tryptophane content was established with the aid of a calibration curve plotted from readings made on aqueous solutions of known concentration. With 0.02 mg. or more of tryptophane the error of analysis did not exceed 1 per cent; for smaller amounts the estimation was less accurate.

Spectral transmittance curves plotted from measurements in the Coleman[1] spectrophotometer showed maximum absorption at 550 mμ. Kynurenine, kynurenic acid, and indolepyruvic acid, all metabolites of tryptophane, developed no color, but indole, skatole, and indolepropionic acid, which may be produced by bacterial action in the intestine, did. In the region of maximum absorption the colors with skatole and indolepropionic acid were qualitatively similar to the color formed with tryptophane; the color with indole was quite different. Colors produced by acetyl-*l*-tryptophane, acetyl-*dl*-tryptophane, phenacetyltryptophane, and methylenetryptophane also showed maximum absorption at about the same wave-length as tryptophane, as had already been observed for acetyltryptophane by Shaw and McFarlane (4). Although these derivatives are not normally found in the body, they resemble peptides closely enough to warrant the assumption that peptides produced metabolically from tryptophane cannot be distinguished from the free amino acid by this method.

No satisfactory procedure was found for estimating indolepyruvic acid in small concentrations, such as might appear transiently in blood and tissues as the result of oxidative deamination of the tryptophane fed. Obviously the urea and non-protein nitrogen analyses conducted instead cannot serve specifically as an index of oxidative α-deamination; ammonia is produced also when kynurenic acid is formed and its production from tryptophane by non-oxidative deamination, *e.g.* hydrolytic, cannot be precluded.

In the tests on blood, the use of potassium oxalate as an anticoagulant and of tungstic acid as a deproteinizing agent did not interfere with the quantitative estimation of tryptophane added before the deproteinization. Blood urea was determined by the method of Koch (5), non-protein nitrogen by a minor modification of the procedure of Keys (6).

Small oral doses of tryptophane in the rabbit and the rat (0.35 gm. per kilo) produced concentrations in the blood which rose to a maximum abruptly and declined to zero again in 5 hours. Unlike the inconsistent and erratic changes after subcutaneous injection, the general trend following oral administration was reproducible. Therefore, in the studies involving the simultaneous estimation of tryptophane, urea, and non-protein nitrogen, male rabbits which had been fasted for 18 hours were given relatively large doses of tryptophane (1 gm. per kilo, suspended in

[1] The double monochromator model was used.

water) by stomach tube. With one exception the dosage caused no ill effects, but larger amounts of tryptophane were sometimes toxic. All blood samples were drawn from the marginal ear vein. The analyses

TABLE I

Changes in Concentration of Tryptophane, Urea, and Non-Protein Nitrogen in Blood of Rabbit after Administration of Tryptophane by Stomach Tube*

Rabbit No.	Weight	Time after feeding	Tryptophane†	Urea N	Non-protein nitrogen	Undetermined N
	kg.	*hrs.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*
7	2.1	0	0	12.9	40.5	27.6
		2	24.4 (3.3)	14.6	49.2	31.3
		4	12.8 (1.7)	22.6	49.2	24.9
		6	2.0 (0.3)	18.1	43.4	25.0
		12	1.6 (0.2)	24.1	48.0	23.7
9	2.6	0	0	15.5	37.4	21.9
		2	19.7 (2.8)	18.6	46.2	24.8
		4	8.4 (1.2)	22.1	46.2	22.9
		6	0	13.6	43.6	30.0
		12	0	25.2	40.0	14.8
9.	2.6	0	0	14.2	41.0	26.8
		2	20.9 (2.9)	17.2	48.8	28.7
		4	17.4 (2.4)	15.5	49.0	31.1
		6	0	14.8	50.0	35.6
		8	0	13.1	47.2	34.1
		10	0	17.6	51.6	34.0
		12	0	17.6		
11	2.5	0	0	14.5	39.8	25.3
		2	18.4 (2.6)	19.5	48.0	25.9
		4	3.2 (0.4)	19.8	48.0	27.8
		6	0	17.1	42.8	25.7
		8	0	19.4	43.6	24.2
		10	0	19.8	43.8	24.0
		12	0	21.5	43.6	22.1
13	2.5	0	2.8 (0.4)	22.6	47.6	24.6
		2	15.7 (2.2)	25.1	52.4	35.1
		4	8.0 (1.0)	20.6	51.4	29.8
		6	0	20.3	49.8	29.5
		8	0	20.3	49.9	29.5
		10	0	22.0	53.0	31.0
		12	0	23.8	49.8	26.0

* The dosage was 1 gm. per kilo of body weight.

† Tryptophane nitrogen is indicated in the parentheses.

(Table I) showed a maximum concentration of tryptophane in 2 hours, less in 4 hours, and little or none in 6; a primary rise in urea to a maximum in the 2 or 4 hour sample, a decline to a minimum in the 6 or 8 hour speci-

men, then a secondary rise; and a concentration of non-protein nitrogen which in most instances reflected the changes in urea and tryptophane.

The depression of urea which accompanied or soon followed the disappearance of tryptophane, to be succeeded later by a secondary rise, made it desirable to determine whether tryptophane is readily withdrawn for a time into the tissues. Rats, which had been fasted for 18 hours, then fed 0.35 to 1.3 gm. of tryptophane per kilo of body weight, were killed with ether, usually 1 to 3 hours later. The gastrointestinal tract was dissected out and washed thoroughly with boiling water to recover the unabsorbed tryptophane. The gastrocnemius muscles, liver, and kidneys were removed and ground separately with sand. The skin was peeled off, together with the fatty layer and the tail, and rinsed free of adhering blood. The rinsings were combined with the blood for analysis with the remainder of the "carcass." The "skin" and "carcass" fractions were ground separately in a meat chopper. Each of the several fractions was extracted eight times with a total of approximately 10 times its weight of boiling water acidified with 1 gm. of trichloroacetic acid per liter to facilitate coagulation. Complete precipitation of protein at this stage required concentrations of trichloroacetic acid high enough to interfere with the tryptophane determination. The small amount of residual protein was therefore removed in a subsequent step, by transferring the extract to a 200 cc. volumetric flask and adding 20 cc. of 25 per cent cupric sulfate and enough 10 per cent calcium hydroxide suspension (usually about 20 cc.) to make the mixture alkaline to litmus. When the volume of the extract was too large, it was first concentrated *in vacuo* to about 100 cc. Several other methods of removing proteins proved to be less satisfactory than this combination.

Large amounts of fatty substances in the skin extracts made precipitation and filtration difficult. Calcium sulfate, which sometimes separated during the assay of the filtrates for tryptophane, was removed by centrifugation just before measuring the color in the photometer. Some of the carcass and skin blanks showed noticeable coloration.

As outlined, analysis accounted fairly completely, but variably, for tryptophane which had been added to carcass tissue (85 to 100 per cent) and to liver (93 to 100 per cent). Recoveries were rather poor from kidney tissue (43 to 54 per cent) and from the skin (44 per cent), and intermediate from leg muscle (52 to 80 per cent). Freezing the tissue afforded no advantage and seemed even to interfere with tryptophane recovery.

Table II shows that in rats fed tryptophane less than a third of the amount absorbed could be detected in the body, even in the 1st hour after the feeding. That none was found in the muscle may be ascribed in part, but not entirely, to the small sample analyzed; this observation

agrees with that of Luck (1) that feeding amino acids induced no significant change in the amino nitrogen content of muscle tissue. Except in a single instance no tryptophane could be found in the kidney. Its concentration in the liver varied widely, being reasonably consistent only in the 3 hour tests, in which it accounted for about a fifth of the total tryptophane found in the body. Either of the two processes ascribed to the liver, deamination to indolepyruvic acid and conversion to kynurenine and kynurenic acid, produces metabolites unresponsive to the tryptophane

TABLE II

Absorption and Distribution of Tryptophane in Rats Fed Tryptophane after 18 Hour Fast

Rat No.	Rat weight	Tryptophane fed	Time for absorption	Tryptophane absorbed	Skin	Tryptophane found*		
						Liver	Carcass†	Total
	gm.	*mg.*	*hrs.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1	275	0	0	0	0	0	0.8	0.8
2	300	0	0	0	0	0	0.3	0.3
11	270	0	0	0	0	0	0.6	0.6
14	190	65	1	44.2	1.7	0.4	11.2	13.3
15	190	65	2	60.5	2.1	0.3	9.0	11.4
13	230	80	1	57.4	0.2	0.9	4.8	5.9
5	260	91	2	82.2	4.6	0.4	10.0	15.0
4	333	91	3	80.4	1.0	2.7	12.1	15.8
3	290	91	10	91	0	0	0.3	0.3
6	290	135	2	129.3	2.7	0.4	22.5	25.6
8	240	135	3	128.8	0.2	2.1	8.9	11.2
10	260	270	3	247.8	2.0	5.0	16.8	24.2‡
9	209	270	6	270	3.3	0	12.7	16.0

* No tryptophane was found in any of the gastrocnemius muscle samples analyzed and, except in one test, none was found in the kidneys. Hence these analyses are not tabulated.

† Includes blood and all of the body except the gastrointestinal tract and the parts analyzed separately.

‡ The kidneys of this animal contained 0.4 mg.

test. Appreciable urinary excretion of kynurenic acid would likely stimulate the kidney to produce ammonia, possibly even from tryptophane, if this were readily available.

The skin retained tryptophane temporarily in amounts representing 13 to 37 per cent of the total in the 1st and 2nd hours, and 2 to 8 per cent in the 3rd. Usually over half of the total tryptophane detected was in the "carcass" fraction, which included the blood. The capacity of these tissues to destroy tryptophane is unknown. Retention of tryptophane as such by the tissues appears to be only temporary and of secondary significance in lowering the tryptophane content of the blood.

The introduction of certain substituents into the α-amino group prevents tryptophane from promoting growth in the rat or yielding kynurenic acid in the rabbit. Obviously, therefore, the substituent must either block metabolism entirely or alter its course profoundly. In the latter event, measurable differences in rate of metabolism could conceivably facilitate interpretation of the observations with tryptophane. With this in mind, methylenetryptophane and phenacetyltryptophane were tested for their influence upon the urea and the non-protein nitrogen content of the blood, as tryptophane had been. Neither of these derivatives promotes growth (7, 8); hence neither would be expected to produce kynurenic acid (methylenetryptophane is known not to do so (9), but phenacetyltryptophane has not been similarly tested). Both compounds are relatively insoluble in acid solution and consequently highly susceptible to precipitation with or adsorption on the tungstic acid precipitate. This probably explains why blood filtrates prepared after the feeding of these derivatives failed to produce a color in the test for tryptophane (Table III), except in the 2nd hour after phenacetyltryptophane administration, when a weak response was noted. Both compounds caused a rise in urea which was not followed by the fluctuations observed consistently in the tests with tryptophane. This seems to argue against failure to undergo absorption and catabolism, though partial excretion unchanged (9) and incomplete catabolism are probable.

The rise in urea noted after feeding phenacetyltryptophane was particularly striking. To determine whether phenylacetic acid could have been liberated and thus involved, phenylacetic acid was fed as the sodium salt. The blood urea did not increase steadily as after the ingestion of phenacetyltryptophane, but showed a rather long initial depression which probably reflected diversion of amino nitrogen for detoxication of the phenylacetic acid. Since the acid was fed as the salt, appreciable diversion for the synthesis of urinary ammonia would seem unlikely. The marked rise in blood urea which followed this depression may reflect readjustments incident to restoration of the normal metabolic state upon release of the stress of mobilizing amino nitrogen for the conjugation.

DISCUSSION

Analysis of the data in the light of previous observations seems to admit of the following interpretation pertaining to animals which excrete kynurenic acid. The early rise in urea and the rapid removal of tryptophane from the blood after tryptophane is fed indicate that the initial steps of its metabolism are begun promptly. This is substantiated in the rat by failure to account for more than a third of the tryptophane absorbed, even

TABLE III

Changes in Concentration of Urea and Non-Protein Nitrogen in Blood of Rabbit Given Methylenetryptophane, Phenacetyltryptophane, or Phenylacetic Acid by Stomach Tube

Rabbit No.	Weight	Substance fed	Time after feeding	Urea N	Non-protein nitrogen	Undetermined N
	kg.		*hrs.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*
12	2.2	Methylenetryptophane (2.34 gm.)	0	14.7	44.8	30.1
			2	15.3	44.8	29.5
			4	16.5	47.0	30.5
			6	15.8	51.6	35.8
			8	22.0	50.8	28.8
			10	22.4	53.4	31.0
			12	22.1	49.4	27.3
14	2.1	Methylenetryptophane (2.22 gm.)	0	18.2	44.0	25.8
			2	16.3	44.4	28.1
			4	18.0	45.0	27.0
			6	20.4	47.2	26.8
			8	21.8	45.8	24.0
			10	19.4	51.6	32.2
			12	21.8	53.4	31.6
15	2.6	Phenacetyltryptophane (2.4 gm.)	0	14.5	46.0	31.3*
			2	20.1	53.2	32.3*
			4	24.5	61.8	37.3
			6	31.4	67.0	35.6
			8	35.3	73.0	37.7
			10	45.5	80.4	34.9
			12	53.1	95.0	41.9
16	3.1	Phenacetyltryptophane (2.2 gm.)	0	21.2	46.4	25.2
			2	23.0	52.4	29.1†
			4	24.7	50.8	26.1
			6	33.0	55.2	22.2
			8	32.2	58.0	25.8
			10	36.8	61.6	24.8
			12	34.1	63.2	29.1
10	2.7	Phenylacetic acid (1.0 gm. as Na salt)	0	12.5	42.8	30.3
			2	11.5	39.4	27.9
			4	8.6	37.0	28.4
			6	8.2	42.2	34.0
			8	10.0	41.2	31.2
			10	18.7	47.1	28.4
			12	20.5	44.5	24.0

* The color produced in the tryptophane test was equivalent to 0.2 mg. of tryptophane N per 100 cc. of blood in the initial period, 0.8 mg. in the 2 hour period.

† The color produced in the tryptophane test was equivalent to 0.3 mg. of tryptophane N per 100 cc. in this period.

in the 1st hour. Earlier studies on rats fed even larger doses of *l*-tryptophane suggest that excretion in the urine was not involved (10). Theoretically, at least, ammonia can be produced metabolically from tryptophane either before or after rupture of the indole ring. The noticeable depression in blood urea which followed the initial peak in every test on tryptophane suggests diversion of nitrogen from urea synthesis, possibly to provide ammonia for the excretion of acidic metabolites, such as kynurenic acid. This supposition is strengthened by the observation that interference with deamination and kynurenic acid synthesis by blocking the α-amino group of tryptophane prevented the fluctuations in blood urea noted with the free amino acid. If the gradual increases observed in the urea nitrogen were produced by the metabolism of the methylene or the phenacetyl derivative fed, initial rupture of the indole ring must be assumed. Operation of the process involved in the synthesis of kynurenine would yield a derivative of kynurenine containing a free amino group. Since rats fed free kynurenine excrete only a portion as kynurenine or kynurenic acid (10), at least one other metabolic path, quantitatively even more important than conversion to kynurenic acid, must be available for kynurenine to follow (10). The only paths obviously closed to kynurenine derivatives such as the ones suggested are those allowing ultimate production of kynurenic acid.

SUMMARY

Analysis of the blood of rabbits at intervals after the ingestion of sizable doses of tryptophane reveals changes in the tryptophane, urea, and non-protein nitrogen contents which begin promptly and follow a characteristic and reproducible sequence. The changes which occur after the ingestion of the methylene or the phenacetyl derivative of tryptophane follow a different sequence.

Suggestions as to the probable course of the metabolism of free tryptophane and the derivatives are made on the basis of these data and on the assumption that the substituent groups alter the course of metabolism chiefly by preventing ready α-deamination and the synthesis and excretion of kynurenic acid.

Analysis of a series of rats killed at intervals after they had been fed tryptophane in amounts too low to cause any considerable excretion showed no tryptophane in the muscle or kidney, but appreciable amounts in the liver, skin, and the remainder of the animal during the first few hours. At no time did the total amount found in the rat represent more than a third of the tryptophane absorbed. This supports the conclusion based on observations with rabbits that at least the initial stages of tryptophane metabolism must proceed rapidly.

BIBLIOGRAPHY

1. Luck, J. M., *J. Biol. Chem.*, **77**, 13 (1928).
2. Johnston, M. W., and Lewis, H. B., *J. Biol. Chem.*, **78**, 67 (1928).
3. Shambaugh, N. F., Lewis, H. B., and Tourtellotte, D., *J. Biol. Chem.*, **92**, 499 (1931).
4. Shaw, J. L. D., and McFarlane, W. D., *Canad. J. Res., Sect. B*, **16**, 361 (1938); *J. Biol. Chem.*, **132**, 387 (1940).
5. Koch, F. C., Practical methods in biochemistry, Baltimore, 2d edition, 121 (1937).
6. Keys, A., *J. Biol. Chem.*, **132**, 181 (1940).
7. Berg, C. P., Rose, W. C., and Marvel, C. S., *J. Biol. Chem.*, **85**, 219 (1929-30).
8. Berg, C. P., and Hanson, H. E., *Proc. Iowa Acad. Sc.*, **41**, 165 (1934).
9. Berg, C. P., *J. Biol. Chem.*, **91**, 513 (1931).
10. Borchers, R., Berg, C. P., and Whitman, N. E., *J. Biol. Chem.*, **145**, 657 (1942).

THE ELECTROPHORETIC MOBILITY OF HUMAN SERUM ALBUMIN AS AFFECTED BY LOWER FATTY ACID SALTS

By GERALD A. BALLOU, PAUL D. BOYER, AND JAMES MURRAY LUCK

(*From the Department of Chemistry, Stanford University, California*)

(Received for publication, March 12, 1945)

In earlier work (1) it was shown that the lower fatty acid anions, when present in low concentrations in solutions of human serum albumin, exerted a marked stabilizing effect against coagulation of the protein by heat. This effect was also observed to increase with increasing chain length of the fatty acid anion. The purpose of this investigation was to ascertain whether any electrostatic association or specific interaction of the lower fatty acid anions with the protein occurred, and to study the effect of chain length on the affinity of the carboxylate ion for the protein. Since combination of such anions, either by polar association with positively charged groups of a protein or by non-polar association, would change the net charge on the protein molecule (2), and since the electrophoretic mobility is a sensitive indicator of the net charge (3), the problem was investigated electrophoretically.

A number of investigators (4–9) have demonstrated by means of mobility measurements a specific interaction of certain buffer anions with dissolved and adsorbed proteins. Specific anion interactions with amylase and taka-diastase have also been reported (10). From titration data, Steinhardt (11) observed a variable affinity of different organic acid anions for protein, and concluded that the specific affinities could not be attributed to electrostatic association alone. As will be seen below, our findings tend to confirm this conclusion.

Lundgren and coworkers (12, 13) and Putnam and Neurath (14) found from electrophoretic mobility measurements a considerable interaction of synthetic detergents with serum and egg albumin. Several factors, however, tend to make the system quite complex when detergents are present. Chief among these (*a*) proteins are readily denatured by detergents such as sodium dodecyl sulfate and the alkyl aryl sulfonates, and (*b*) except in very dilute solutions, the detergents are present not in true solution but in the state of micelles. Another factor which may influence considerably the relationship between mobility and detergent concentration is the marked variation in viscosity with increase in detergent concentration. As is well known, the electrophoretic mobility is inversely proportional to the viscosity of the medium.

Instead of a study of the effects of the different anions on the mobility-pH relationship at various ionic strengths (4, 5, 7, 8), the influence of concentration of the anion on the mobility of the protein at a constant ionic strength of 0.2 and pH of 7.7 was determined.

It is improbable that any denaturation of the protein was produced by the fatty acid anions used (15), or that any appreciable micelle formation of the anions occurred within the concentration ranges herein studied.

EXPERIMENTAL

Crystalline human serum albumin, obtained from the Plasma Fractionation Laboratory, Harvard University, was used. The sodium salts of butyric, caproic, heptoic, and caprylic acids, either in the anhydrous state or in solution, were used in the preparation of the solutions.

For each run 1.8 liters of buffer-salt solution were prepared; these contained the desired proportions of sodium chloride, sodium salt of the fatty acid, and phosphate buffer. The pH of the solution, after being diluted to volume, was finally adjusted by the dropwise addition of 3 N NaOH to 7.70 with the aid of a Beckman pH meter. In the ionic strength calculations the ratio $(HPO_4^=)/(H_2PO_4^-)$ was calculated to be 10 at pH 7.7.

0.5 gm. of the crystalline albumin was dissolved in 100 ml. of the buffer-salt solution, and the pH of this solution was carefully adjusted to 7.70. This volume of approximately 0.5 per cent protein was then dialyzed against the remaining 1.7 liters of buffer-salt solution until equilibrium was attained. The final pH, conductivity, and viscosity measurements were made on the protein solution after dialysis. The pH was determined at room temperature, while the conductivity and viscosity measurements were made at 0.6°, the temperature of the electrophoresis bath.

The Longsworth modification (16) of the electrophoresis apparatus was used to obtain the mobility data in this work. Photographs of the initial and final boundaries were made by the scanning method of Longsworth (16). The potential gradients used for all of the runs came within the range of 4 to 6 volts per cm. The time intervals between initial and final photographs varied from 2.5 to 4 hours. In the final photographs, owing to the low protein concentration and high ionic strengths, the δ and ϵ boundaries were practically negligible. The ascending and descending boundaries were quite symmetrical and indicated a high state of homogeneity. Only the mobilities of the descending boundaries were used in these studies.

All mobilities were corrected to the viscosity of water (2, 17). The relative viscosities varied from 1.020 to 1.123 for the protein salt solutions, and hence the largest correction was about 12 per cent for the solution containing 0.1 M sodium caprylate. The relative viscosity-salt concentration curves were simple straight line functions.

RESULTS AND DISCUSSION

Preliminary runs indicated that, in addition to the carboxylate ion, a variable phosphate concentration caused a change in mobility, so that it was deemed necessary before using phosphate as a buffer to investigate the influence of a variable phosphate concentration at an ionic strength of 0.2 and pH of 7.69 ± 0.03. Fig. 1 illustrates the effect on the mobility of albumin of variations in the total phosphate concentration from 0.0072 M to 0.072 M. At 0.0072 M phosphate, 0.18 M sodium chloride was present, while no sodium chloride was present at 0.072 M phosphate. The increase in mobility, corrected to the viscosity of water, and in sq. cm. sec.$^{-1}$ volt^{-1}, amounted to about 30 per cent. Evidently there is a considerable specific interaction of the divalent phosphate ion with the protein. Of the total

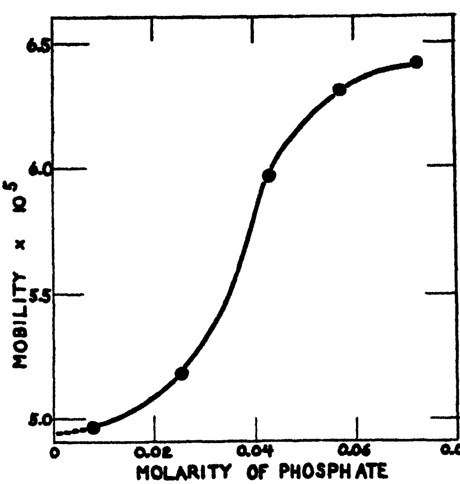


FIG. 1. The effect of phosphate on the electrophoretic mobility of human serum albumin.

phosphate present, 91 per cent consisted of $HPO_4^=$. An increase in protein mobility due to phosphate interaction has also been observed by Moyer (5) and by Longsworth (9).

In view of these data for phosphate it was our intention to use, in the presence of the carboxylate ions, a phosphate concentration low enough to provide some buffering action and yet not high enough to complicate any results attributable to association of the fatty acid anions. Fig. 2 presents the results obtained with caprylate, heptoate, caproate, and butyrate. The phosphate concentration was 0.025 M in all runs, while the sum of the concentrations of sodium chloride and the sodium salt of the fatty acid was 0.13 M, thus yielding a constant ionic strength of 0.2. The approximate mole ratio of carboxylate ion to albumin, at a carboxylate concentration of

0.10 M, was 1400. Owing to the manner of preparation of the protein-buffer solutions, the carboxylate ion concentration in these experiments is essentially that of the free fatty acid anions in equilibrium with the protein-anion complex.

These curves demonstrate clearly a specific interaction of these anions with the albumin, and an increasing affinity in ascending from butyrate to caprylate. This increase in affinity corresponds to the increase in thermal stability previously noted (1). If the electrostatic attraction between the anion and the positive groups of the protein is similar for the different carboxylate ions, as it may well be since the dissociation constants of the acids are very nearly the same, then the observed difference in association

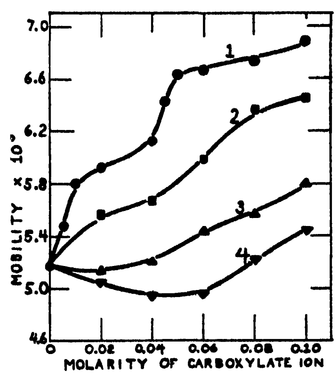


FIG. 2. The effect of lower fatty acids on the electrophoretic mobility of human serum albumin. The curves represent addition of (Curve 1) sodium caprylate, (Curve 2) sodium heptoate, (Curve 3) sodium caproate, and (Curve 4) sodium butyrate.

of the anions with the protein may be attributed to the increasing length of the hydrocarbon chain (11) and probably to increasing van der Waals forces.

The inflection in the caprylate curve, which is apparent but less marked in the heptoate curve, would seem to indicate groups or positions on the protein ion having different affinities for the same carboxylate ion. However, the causative factor for the initial dip in the caproate and the butyrate curves, which may be due to a combined effect of phosphate and the carboxylate anion, may also be responsible for the inflections in the caprylate and heptoate curves.

Interesting speculations arise when one considers that the electrostatic and probably sole type of interaction between the divalent phosphate ion and the positive groups of the protein may increase the net charge on the complex by an association of an $HPO_4^{=}$ ion with two positive charges on the

protein, or by association of $HPO_4^=$ with one positive charge on the protein, thus leaving a negative charge on the phosphate free. The resultant net charge on the complex would be the same in either case. However, if the $HPO_4^=$ ion, doubly associated, were to be displaced by a singly charged anion of high affinity for the protein and were caused to associate in the second manner, the net charge on the complex would be increased with no loss in phosphate to the complex.

This may be the explanation for the results obtained in Fig. 3 in which the mobilities for various combinations of caprylate and phosphate are presented. The pH and ionic strengths were the same as before; *i.e.*, 7.7 and 0.2. It will be noted that the mobility rises and levels off at a value appreciably greater than that for 0.06 M caprylate in Fig. 2 or for

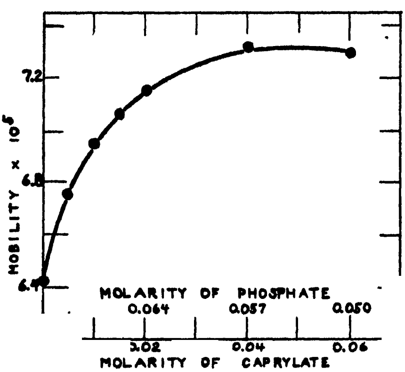


FIG. 3. The effect of caprylate and phosphate on the electrophoretic mobility of human serum albumin.

0.072 M phosphate in Fig. 1. Another and perhaps no less plausible explanation may be that the summation effect in Fig. 3 is caused by the increasing non-polar association of the hydrocarbon chain of the caprylate ion with non-polar chains or portions of the protein molecule. This effect may also be responsible for the slow rise in mobility between 0.05 M and 0.10 M caprylate in Fig. 2.

One run was made in the presence of 0.054 M phosphate and 0.05 M butyrate in which a mobility of 6.0×10^{-5} sq. cm. sec.$^{-1}$ volt^{-1} was obtained. If this mobility is compared with that for the caprylate-phosphate mixture of the same composition, namely 7.3×10^{-5} sq. cm. sec.$^{-1}$ volt^{-1}, and with 6.25×10^{-5} sq. cm. sec.$^{-1}$ volt^{-1} for 0.054 M phosphate and 0.05 M chloride, there appears the same phenomenon that is illustrated by the butyrate curve of Fig. 2; that is, an apparent decrease in net charge on the complex caused by the butyrate.

SUMMARY

1. The effects of the butyrate, caproate, heptoate, caprylate, and phosphate ions on the electrophoretic mobility of human serum albumin were studied.

2. Evidence of an anion-albumin association, which increases with increase in chain length, is presented.

We are indebted to the Plasma Fractionation Laboratory, Harvard University, for the albumin used in these studies and to Professor Carl Noller of Stanford University for the heptoic acid which was employed.

This work was done under a contract recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Stanford University.

BIBLIOGRAPHY

1. Ballou, G. A., Boyer, P. D., Luck, J. M., and Lum, F. G., *J. Biol. Chem.*, **153**, 589 (1944); *J. Clin. Invest.*, **23**, 454 (1944).
2. Abramson, H. A., Moyer, L. S., and Gorin, M. H., Electrophoresis of proteins and the chemistry of cell surfaces, New York (1942).
3. Abramson, H. A., Gorin, M. H. and Moyer, L. S., *Chem. Rev.*, **24**, 345 (1939).
4. Smith, E. R. B., *J. Biol. Chem.*, **108**, 187 (1935).
5. Moyer, L. S., *Tr. Faraday Soc.*, **36**, 248 (1940).
6. Moyer, L. S., and Moyer, E. Z., *J. Biol. Chem.*, **132**, 373 (1940).
7. Sookne, A. M., and Harris, M., *J. Res. Nat. Bur. Standards*, **23**, 299 (1939).
8. Davis, B. D., and Cohn, E. J., *J. Am. Chem. Soc.*, **61**, 2092 (1939).
9. Longsworth, L. G., *Ann. New York Acad. Sc.*, **41**, 267 (1941).
10. Ballou, G. A., and Luck, J. M., *J. Biol. Chem.*, **135**, 111 (1940); **139**, 233 (1941).
11. Steinhardt, J., *Ann. New York Acad. Sc.*, **41**, 287 (1941).
12. Lundgren, H. P., Elam, D. W., and O'Connell, R. A., *J. Biol. Chem.*, **149**, 183 (1943).
13. Lundgren, H. P., and O'Connell, R. A., *Ind. and Eng. Chem.*, **36**, 370 (1944).
14. Putnam, F. W., and Neurath, H., *J. Am. Chem. Soc.*, **66**, 1992 (1944).
15. Rice, R. G., Ballou, G. A., Boyer, P. D., Luck, J. M., and Lum, F. G., *J. Biol. Chem.*, **158**, 609 (1945).
16. Longsworth, L. A., *Chem. Rev.*, **30**, 323 (1942).
17. Daniel, J., *J. Gen. Physiol.*, **16**, 459 (1933).

BIOLOGICAL ESTIMATION OF THE THIAZOLE AND PYRIMIDINE MOIETIES OF VITAMIN B_1

BY H. G. OBERMEYER AND LING CHEN

(*From the Research Laboratories of Merck and Company, Inc., Rahway, New Jersey*)

(Received for publication, March 23, 1945)

Fungus growth methods for estimating either vitamin B_1 thiazole or vitamin B_1 pyrimidine[1] have been used by Robbins and Kavanagh (1), Kavanagh (2), and Krampitz and Woolley (3), and a fermentation stimulation method has been described by Schultz, Atkin, and Frey (4). A procedure utilizing the synthesis of vitamin B_1 by yeast from thiazole and pyrimidine has been used in this laboratory to estimate minute quantities of these moieties. By suitable differential procedure, thiamine, thiazole, and pyrimidine in food materials may be estimated individually. The procedure and certain of its limitations are described in this paper.

Method

25 gm. of patent flour were introduced into a clean dry 500 ml. volumetric flask. 1 gm. of fresh bakers' yeast (Fleischmann, in water suspension) and water to 40 ml. were added. The system was then mixed by vigorous swirling into a smooth, lump-free batter, stoppered loosely, and allowed to ferment 18 hours (overnight) at room temperature.

Flour was used as a practical expedient to furnish yeast nutrients and to avoid the need for a continuous shaker. The flour-water ratio used should permit the formation of a batter mobile enough for thorough mixing yet viscous enough to prevent settling of the yeast and flour solids.

The materials being tested were added to assigned flasks as solutions or suspensions just before the balance of the water which was used to rinse down the necks. Sixteen of these flasks were usually run in one series.

After fermentation, the batter was dispersed in 250 ml. of 0.05 N sulfuric acid, and heated 30 minutes in a boiling water bath. The flasks were swirled at least once every minute for the first 5 minutes of the heating until the starch gelatinized. Any unfermented controls were prepared at this point. In these controls, the acid was introduced before the yeast to prevent synthesis. When cooled, the suspensions were neutralized to pH 4.5 with sodium acetate solution. Then 0.5 gm. of clarase in 10

[1] Throughout this paper, unless otherwise stated, the term thiazole (I) refers to 4-methyl-5-hydroxyethylthiazole and pyrimidine (II) refers to 2-methyl-5-ethoxymethyl-4-aminopyrimidine.

ml. of water was added and the suspensions incubated overnight at 38°. This treatment was found to extract and hydrolyze cocarboxylase efficiently. The assay for thiamine was then made by means of the thiochrome method with the Decalso purification recommended by Hennessy (5).

To determine the increase in thiamine produced by yeast synthesis, it was necessary to determine first the thiamine in the unfermented control, contributed by the flour, yeast, and the material being tested. The difference in the vitamin B_1 content between the fermented and unfermented samples was regarded as the amount of vitamin B_1 synthesized by the yeast. This was assumed to be due to the presence of equimolar quantities of a thiazole derivative and a pyrimidine derivative which are capable of

TABLE I

Efficiency of Conversion and Recovery

Sample and treatment	Observed vitamin B_1 (as hydrochloride) per sample	Theory
	γ	*per cent*
Control, unfermented	18.9	
" fermented	18.2	
100 γ vitamin B_1 hydrochloride, unfermented	118.0	99
100 " " " " fermented	115.1	96
50 " (I), fermented	18.0	
50 " (II), "	18.5	
30 " (I) and 30 γ (II) (0.1796 micromole), fermented	76.7	96
50 " " " 50 " " (0.2994 "), "	119.2	100
100 " " " 100 " " (0.5988 "), "	213.4	97
50 " " (0.3497 micromole) and 100 γ (II), fermented	135.1	99
100 " " and 50 γ (II) (0.2994 micromole), "	115.9	97

yielding vitamin B_1 upon combination. Amounts of either moiety in excess of equimolar proportions were estimated by adding increments of the limiting moiety to duplicate systems before the fermentation until a maximum production of vitamin B_1 was obtained.

Efficiency—The efficiency of this procedure in converting the thiamine moieties (I) and (II) into vitamin B_1 may be seen in the data of Table I, in which the molar concentration of the limiting moiety is indicated.

The recovery of added thiamine and the efficiency of the yeast synthesis ranged between 95 and 100 per cent under these conditions. Thus, at these thiamine levels, a correction factor was not required. The data in Table I indicate that slight excesses of either moiety had no effect upon the efficiency of the conversion under these conditions and that the yeast did not synthesize either (I) or (II).

Specificity—Although (I) and (II) are the moieties expected from thiamine degradation, they are not the only ones which can be utilized in the yeast synthesis of vitamin B_1. Fink and Just (6) tested different thiazole and pyrimidine derivatives with varied types of yeast and observed that several derivatives could be utilized by the yeasts. Their results are in agreement with those of Schultz (7) who found that the physiological activity in chemically prepared analogues of thiamine was retained when the alkyl groups were changed in position 2 of the pyrimidine and position 4 of the thiazole; changes in positions other than these greatly reduced the vitamin B_1 activity.

Several thiazole and pyrimidine derivatives[2] were available and were tested with the yeast synthesis procedure to determine whether any were

TABLE II

Yeast Synthesis with Varied Thiazole and Pyrimidine Derivatives

Sample containing 50 γ thiazole and 50 γ pyrimidine designated	Observed vitamin B_1 (as hydrochloride) per sample	Theoretical
	γ	*per cent*
Control	18.5	
I) and (II) (0.2994 micromole)	112.5	95
" " (III)	18.0	
" " (IV)	18.0	
" " (V) (0.2762 micromole)	80.5	67
" " (VI)	17.5	
" " (VII)	18.0	
" " (VIII)	18.0	
" " (IX)	18.5	
X) (0.2703 micromole) and (II)	76.0	63
XI) and (II)	17.5	
X) (0.2703 micromole) and (V)	70.0	57

utilized by the yeast to form a thiamine analogue capable of being converted to a fluorescing substance upon alkaline oxidation but being devoid of vitamin B_1 activity in itself. The following thiazole and pyrimidine derivatives were tested: 2-methyl-4-amino-5-methylsulfonic acid pyrimidine (III), 2-methyl-4-hydroxy-6-ethoxymethylpyrimidine (IV), 2-ethyl-4-amino-5-ethoxymethylpyrimidine (V), 2-ethyl-4-hydroxy-5-ethoxymethylpyrimidine (VI), 4-hydroxy-6-methyl-5-methylpyrimidylethyl acetate (VII), 2-aminopyrimidine (VIII), 2-amino-4-chloropyrimidine (IX), 4-methyl-5-β-acetoxyethylthiazole (X), and 3-iodomethyl-4-methyl-5-β-hydroxyethylthiazole (XI). The results are shown in Table II. The molar concentrations of the limiting moiety are indicated.

[2] Kindly supplied by Dr. G. A. Stein.

These data show that yeast utilized the combinations (I) and (V), (X) and (II), and (X) and (V) to produce compounds capable of being converted to a "thiochrome." It is probable that other combinations of a number of thiazole and pyrimidine derivatives can be utilized by yeast in this way. For this reason, the precise configuration of the thiazole and pyrimidine moieties present in unknown materials cannot be indicated by this method.

Deutsch (8) has observed that thiazole (I), pyrimidinesulfonic acid, oxychlorothiamine, certain phosphates, and other substances interfere in the measurement of pyrimidine (II) by the yeast fermentation method in that they affect the rate of fermentation. Interference in the estimation of these moieties due to changes in the rate of fermentation has not been encountered in the resynthesis method.

It was of interest to determine whether the analogues of thiamine produced by yeast had physiological activity. A quantity of the compound formed by yeast from (X) and (V) was prepared by the non-proliferating procedure described by Van Lanen *et al.* (9). After thorough washing, the yeast was heated in dilute acid solution, neutralized, and digested with clarase. The filtrate was assayed by the thiochrome procedure. Inspection of the aqueous layer following the oxidation and isobutanol extraction showed that some "cocarboxylase" had escaped hydrolysis. The clarase treatment was repeated until the filtrate was freed of "cocarboxylase." This solution was tested for vitamin B_1 activity by curative and prophylactic tests in rats and was found to contain the same activity as indicated by the thiochrome test. This compound would correspond to the 2-ethylacetoxy vitamin described by Schultz (7) unless during its preparation the acetoxy group had been converted to a hydroxyl group, in which case it would correspond to the 2-ethyl analogue reported by Stein *et al.* (10) to have physiological activity. Similarly, other solutions containing the compounds synthesized by yeast from known (I) and (II) and from the degradation products of thiamine hydrochloride (heated in water at pH 8.0) were observed to have the same activity in rats as like amounts of thiamine hydrochloride. This is in agreement with the report of Fink, Just, and Hock (11) who found that biosynthetic vitamin B_1 was active in rats.

Application—The procedure was used to estimate the increased amounts of thiazole and pyrimidine moieties in food materials when subjected to thermal treatments of such nature as to cause partial destruction of the thiamine. The food materials were assayed for thiamine before and after the thermal treatment and the difference was regarded as the amount of thiamine destroyed. Assuming the destruction consisted of simple cleavage or hydrolysis, the theoretical amounts of (I) and (II) involved were calculated, as indicated in Table III. The thermal treatment varied with

the material. An aqueous solution of thiamine hydrochloride was adjusted to pH 8.0 with borate buffer and was kept at 55° for 4 days. During this treatment, 85 per cent of the thiamine was destroyed. Aliquots of this solution were used in the resynthesis test. The autoclaved yeast was prepared by autoclaving 5 hours at 120° and subsequently drying. 5 gm. portions of the dried preparation were mixed directly with the flour for the resynthesis test. The bread referred to in Table III was the white enriched variety and was baked 30 minutes in an oven at 220°. An entire loaf was dispersed in 0.05 N H_2SO_4 in the Waring blendor, heated, neutralized, digested with clarase, and filtered. Portions of the filtrate were used for the resynthesis test. The flour sample referred to was enriched flour which had been kept at 55° for 3 weeks in a closed container. This flour replaced the control flour in the resynthesis test. The sugar sample was prepared by mixing commercial cane sugar with thiamine hydrochloride

TABLE III

Resynthesis of Thiamine Degradation Products by Yeast

Thiamine (as the hydrochloride) in micrograms per sample.

Type of decomposition	Thiamine known to be present		Increase in vitamin B_1 after fermentation	Increase in vitamin B_1 after fermentation with excess (I)	Increase in vitamin B_1 after fermentation with excess (II)	Per cent of that involved in decomposition	
	Active	Destroyed				(I)	(II)
Thiamine solution	42	92	19	54	16	21	59
Autoclaved yeast	17	145	45	42	80	55	29
Bread	167	30	10	9	20	67	30
Flour	65	60	15	22	17	28	37
Sugar	40	94	7	23	7	8	24

(1 mg. per 10 gm. of sugar) and maintaining it at 38° for 10 months. Aliquots of an aqueous solution of this mixture were used in the resynthesis test. The efficiency of the vitamin B_1 synthesis was checked when each of the materials referred to in Table III was included in the fermenting system. The increments of (I) and (II) used in each case were 50 and 100 γ.

These data indicate that substantial amounts of biologically available thiazole and pyrimidine remain in food material in which thiamine has undergone partial decomposition. The amounts of (I) and (II) available to the yeast differed in the different food products. It appeared that in the samples in which the thiamine degradation was induced by relatively low temperatures and extended time less thiazole than pyrimidine remained. In the materials processed by higher temperatures, more thiazole than pyrimidine remained. In view of the possible significance of the intestinal

synthesis of thiamine, residual thiazole and pyrimidine moieties should be considered in the designing and interpretation of feeding experiments in which vitamin B_1 effects are to be studied.

The authors are grateful to Dr. G. A. Emerson for her valuable contributions and to Miss Betty Wurtz who assisted her in performing the rat assays and to Dr. T. J. Webb, Dr. Klaus Unna, and Dr. J. C. Keresztesy for their helpful suggestions.

SUMMARY

A procedure based upon the synthesis of vitamin B_1 by bakers' yeast has been used to estimate the biologically available thiazole and pyrimidine moieties in thiamine degradation products. By suitable differential procedure, thiamine and the thiazole and pyrimidine moieties in mixtures may be estimated individually without interference from other fermentation-stimulating substances. The activity of several derivatives of thiazole and pyrimidine was determined. Use of the procedure has indicated that substantial amounts of the thiazole and pyrimidine moieties may occur in food products and that the amounts varied in different types of material.

BIBLIOGRAPHY

1. Robbins, W. J., and Kavanagh, F., *Am. J. Bot.*, **25**, 229 (1938).
2. Kavanagh, F., *Bull. Torrey Bot. Club*, **69**, 669 (1942).
3. Krampitz, L. O., and Woolley, D. W., *J. Biol. Chem.*, **152**, 9 (1944).
4. Schultz, A. S., Atkin, L., and Frey, C. N., *J. Biol. Chem.*, **136**, 713 (1940).
5. Hennessy, D. J., *Cereal Chem. Bull.*, **2**, 25 (1942).
6. Fink, H., and Just, F., *Biochem. Z.*, **311**, 287 (1942); **313**, 39 (1942).
7. Schultz, F., *Z. physiol. Chem.*, **265**, 113 (1940).
8. Deutsch, H. F., *J. Biol. Chem.*, **152**, 431 (1944).
9. Van Lanen, J. M., Broquist, H. P., Johnson, M. J., Baldwin, I. L., and Peterson, W. H., *Ind. and Eng. Chem.*, **34**, 1244 (1942).
10. Stein, G. A., Sampson, W. L., Cline, J. K., and Stevens, J. R., *J. Am. Chem. Soc.*, **63**, 2059 (1941).
11. Fink, H., Just, F., and Hock, A., *Ber. chem. Ges.*, **75**, 2101 (1942).

STUDIES IN CARBOHYDRATE METABOLISM

IV. THE TURNOVER OF GLYCOGEN IN THE LIVER AND CARCASS OF RATS FED GALACTOSE*

By DeWITT STETTEN, Jr.

With the Technical Assistance of Esther Rothstein

(*From the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York*)

(Received for publication, April 2, 1945)

In an earlier paper in this series observations were reported on the rate at which glycogen in the liver and carcass of rats was being replaced by freshly synthesized glycogen while the animals were maintained at constant weight (1). The rats used in those experiments were fed a diet containing 60 per cent of glucose. From determination of the rate at which deuterium was incorporated into glycogen while the body fluids of the rats were enriched with D_2O, it could be shown that the half life of glycogen was approximately 1.0 day in the liver and 3.6 days in the carcass, and that only about 0.44 gm. of freshly synthesized glycogen was deposited each day in the entire body of an adult female rat under these circumstances.

The present report deals with similar experiments in which *d*-galactose was the sole dietary carbohydrate. Galactose was selected as the test sugar because earlier workers had found it to be a slow glycogen former (2), and also because, on structural grounds, it appeared doubtful whether galactose could be converted into glucose without prior rupture of the hexose chain into triose fragments. We have previously indicated the reasons for our expectation that glycogen, synthesized from fragments smaller than hexose in an animal whose body fluids contain D_2O, should, when isolated, approach in isotope concentration 66 per cent of that in the body water (1). Experimentally, deuterium concentrations of 56 to 57 per cent of that in the body water were obtained in the liver glycogen in a short time when lactic acid (3) or alanine[1] served as precursor. If, starting from galactose, glycogenesis proceeds exclusively through lactate, similarly high isotope concentrations might be anticipated in the glycogen deposited.

Rats were maintained on a 60 per cent galactose diet for 1, 2, 4, and 8 days after the initiation of D_2O administration, and the rise in deuterium concentration in the glycogen of liver and carcass was observed. From the analytical values obtained (Table I) it is clear that in both liver and

* This work was carried out with the aid of grants from the Nutrition Foundation, Inc., and the Josiah Macy, Jr., Foundation.

[1] Stetten, D., Jr., and Klein, B., unpublished.

carcass the isotope concentration which was approached asymptotically in the glycogen lies closer to 30 per cent than it does to 60 per cent of that in the body water. The deuterium concentration of the liver glycogen attained very nearly its maximal value in 2 days and did not rise appreciably in the succeeding 6 days of observation. Indeed, after only 1 day the concentration of isotope in the glycogen from the liver had risen to a value of more than half of the maximal level, indicating a half life of 1 day or less, or a turnover of 70 per cent or more per day (Equation 3 (1)). The distribution of the values is not suitable for a more precise estimate of the rate of turnover.

The concentration of isotope in the glycogen of the carcass was apparently still rising slowly after 8 days. Extrapolation from the 4 and 8 day

TABLE I

Uptake of Deuterium into Glycogen of Liver and Carcass

A diet containing galactose as the only hexose was administered to pairs of adult rats for 1, 2, 4, and 8 days after the body fluids had been enriched with deuterium oxide. Glycogen was isolated from the livers and carcasses and its deuterium concentration compared with that of the body fluid.

Duration	Concentration of deuterium				
	Analytical figures			Recalculated values	
	Body water	Liver glycogen	Carcass glycogen	Liver glycogen	Carcass glycogen
days	*atom per cent D*	*atom per cent D*	*atom per cent D*	*per cent of body water*	*per cent of body water*
1	1.53	0.298	0.097	19.5	6.3
2	1.65	0.432	0.206	26.2	12.5
4	1.31	0.395	0.292	30.2	22.3
8	1.31	0.369	0.383	28.2	29.2

values (Equation 2 (1)) indicates that the maximal value, achieved only in infinite time, would be approximately 32 per cent of the deuterium concentration in the body water. The rate of turnover of muscle glycogen, k (Equation 1 (1)), has been calculated by the method of least squares as 30 per cent replaced per day, which makes the half life of muscle glycogen about 2.3 days.

Whereas the *fraction of all the glycogen* replaced each day in liver and in carcass when galactose was fed was as large as, or larger than, the corresponding fraction when glucose was fed (1), the actual *weight of glycogen* newly synthesized and deposited each day in the body of the rat was undoubtedly less when galactose was fed. This apparent discrepancy arises from the fact that the quantity of glycogen present both in carcass and in liver is appreciably less when galactose rather than glucose is the

sugar of the diet. Thus, when glucose was fed, the glycogen recovered from the liver averaged 547 mg., from the carcass 331 mg., whereas, after feeding of galactose, an average of only 74 mg. of glycogen was found in the liver, 211 mg. in the carcass. In view of the uncertainty of the rate of turnover of the liver glycogen referred to in the previous paragraph, we do not feel justified in calculating the precise weight of glycogen replaced per day in rats eating galactose.

The fact that the deuterium concentration in the glycogen of neither liver nor carcass shows any tendency to approach the expected maximum of 66 per cent requires the conclusion that a portion of the hydrogen of the glycogen synthesized from galactose was neither derived from nor in equilibrium with the hydrogen of the body water. In view of our previous demonstration that the hydrogen of glycogen synthesized from lactate is almost exclusively either derived from or in equilibrium with that of the body water (3), it may be concluded that in the conversion of galactose to glycogen lactate is not a major intermediate. Regardless of whether glycogen was synthesized from glucose or from galactose, only about one-half of the carbon-bound hydrogen in each newly formed glycogen molecule was derived from the body water. The other half of the stable hydrogen in the glycogen formed must have been derived from hydrogen initially bound to the hexose of the diet.

It follows that galactose may be converted into glycogen in the animal without labilization of some of its hydrogen. Three possible pathways suggest themselves and there are no clues in the present data favoring any one of these. (*a*) Epimerization about carbon atom 4 may occur without rupture of the hexose chain. (*b*) Rearrangement of the hexose molecule may occur through the intermediate formation of a cyclohexitol (4), as suggested by Fischer. (*c*) Galactose may be split into fragments which immediately recondense to form glucose.

When rats are fed galactose in the quantities administered in the present experiment, this sugar appears in considerable quantities in the urine. About 8 per cent of galactose was present constantly in the urine and the urine volumes were large, about 100 cc. per rat per day. The isolation of urinary galactose was accomplished by its oxidation to galactonic acid and subsequent condensation with *o*-phenylenediamine to the readily crystallized benzimidazole derivative (5). This material isolated from urine contained only 0.010 atom per cent D. The water from the same sample of urine contained 1.33 per cent D_2O. This is in striking contrast to the urinary glucose of the diabetic rat in which, under similar circumstances, very considerable concentrations of deuterium were found (6).

The absence of deuterium from the urinary galactose serves as a strong argument against the possibility of biologically activated exchange of

carbon-bound hydrogen atoms in the hexose molecule. It is always difficult to establish with certainty that such exchange does not occur. The stability of the carbon-bound hydrogen atoms of glycogen and of glucose has, under certain selected conditions, already been demonstrated. Thus, whereas 34 per cent of the hydrogen of glycogen, corresponding roughly to the hydroxyl hydrogen, exchanges with water on simple solution, no deuterium can be made to exchange with the remaining 66 per cent even on prolonged boiling with alkali and D_2O (1). Once the isotope in the 34 per cent of exchangeable positions has been eliminated, no further isotope is lost on repeated treatment of deuterio glycogen with aqueous alkali. Glycogen samples have been isolated after feeding of lactate which contained concentrations of isotope in fair agreement with predicted values (3). Deuterio glucose has been isolated, as the pentaacetate, from the urine of a diabetic rat receiving D_2O, and conversion of this pentaacetate to glucosazone resulted in the loss of exactly one-seventh of the deuterium, corresponding to the one carbon-bound hydrogen atom displaced in the process. After prolonged heating of glucose in heavy water, the glucose pentaacetate isolated contained no significant amount of deuterium (6).

To these lines of evidence should now be added the observation that on passage through the body of a rat whose body fluids contain D_2O galactose acquires no stably bound deuterium. It has earlier been argued, on similar grounds, that the failure to find any deuterium in those fatty acids which the animal does not synthesize, linoleic and linolenic, indicates the animal's inability to introduce deuterium into fatty acids by exchange, and hence the occurrence of deuterium in other fatty acids under the same circumstances indicates synthesis, or at least chemical reaction (7). This argument may now be applied to the present situation. Whereas galactose is apparently not synthesized to any large extent by the male rat, it is certainly not a highly abnormal metabolite. The fact that the rats in the present experiment excreted galactose devoid of carbon-bound deuterium may therefore be taken as strong evidence against the occurrence of exchange of carbon-bound hydrogens of hexoses in the animal body. It gives additional weight to the interpretation of the positive finding of deuterium in urinary glucose under various circumstances, and permits, with reasonable certainty, the use of the deuterium concentration in urinary glucose as a measure of glucose synthesis.

EXPERIMENTAL

The diet used consisted of galactose (Smaco) 60 parts, casein (Labco, vitamin-free) 22 parts, yeast powder 6 parts, salt mixture (8) 6 parts, and roughage (Celluration) 6 parts. The purity of the galactose was confirmed

by measurement of its specific rotation after mutarotation; $[\alpha]_D^{25} = +80.8°$.

Two white male rats of the Sherman strain were used in each of four experiments. The rats weighed 195 to 200 gm. initially and were placed on the experimental diet for a few days until approximately constant weight was achieved. Diet and drinking water were allowed *ad libitum*.

Elevation of the D_2O concentration in the body fluids was abruptly effected (1) by the subcutaneous injection of 99.5 per cent D_2O, 1 cc. per 100 gm. of body weight, and was maintained by replacement of the drinking water by 1.5 to 1.85 per cent D_2O. Rats were killed 1, 2, 4, and 8 days after initiation of D_2O administration. Isolations of glycogen were carried out by previously described methods (1).

Urine was collected under toluene and analyzed at frequent intervals for reducing sugar. From the pooled urine collected during the period of D_2O administration, a 25 cc. aliquot, containing 2 gm. of galactose, was concentrated *in vacuo* to a syrup. The residue was taken up in methanol, filtered, and oxidized with I_2 in methanolic KOH (5). The potassium galactonate obtained was condensed with *o*-phenylenediamine according to the procedure of Moore and Link (5) and the benzimidazole derivative repeatedly recrystallized from water.

SUMMARY

When rats were fed galactose instead of glucose as the sole dietary sugar, less glycogen was recovered from their tissues, especially from their livers. The rate at which deuterium was incorporated into this glycogen from D_2O in the body fluid was at least as rapid as when glucose was fed. From the maximal isotope concentrations in the glycogen it could be shown that the animal can convert galactose into glycogen without labilization of all of the carbon-bound hydrogen.

The galactose excreted in the urine when galactose is fed to male rats receiving D_2O is essentially free of deuterium. This finding provides confirmation of the non-exchangeable nature of the carbon-bound hydrogen of hexose.

BIBLIOGRAPHY

1. Stetten, D., Jr., and Boxer, G. E., *J. Biol. Chem.*, **155**, 231 (1944).
2. Voit, C., *Z. Biol.*, **28**, 245 (1891).
3. Boxer, G. E., and Stetten, D., Jr., *J. Biol. Chem.*, **155**, 237 (1944).
4. Fischer, H. O. L., *Harvey Lectures*, in press (1944–45).
5. Moore, S., and Link, K. P., *J. Biol. Chem.*, **133**, 293 (1940).
6. Stetten, D., Jr., and Boxer, G. E., *J. Biol. Chem.*, **156**, 271 (1944).
7. Bernhard, K., and Schoenheimer, R., *J. Biol. Chem.*, **133**, 713 (1940).
8. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **15**, 311 (1913).

THE ADSORPTION OF PHOSPHATES BY ENAMEL, DENTIN, AND BONE

I. ADSORPTION TIME STUDIED BY MEANS OF THE RADIOACTIVE ISOTOPE*

By E. GUNNAR JOHANSSON, MARLENE FALKENHEIM, AND HAROLD CARPENTER HODGE

(From the Division of Dental Research and the Department of Biochemistry and Pharmacology, School of Medicine and Dentistry, The University of Rochester, Rochester, New York)

(Received for publication, March 17, 1945)

Studies of the relative abilities of powdered bone, dentin, and enamel to adsorb various ions, *e.g.* phosphate (1, 2), from aqueous solutions have shown that, for short exposures of the powdered tissues to the solutions, bone adsorbs more than dentin and dentin adsorbs more than enamel. Since the calcified tissues are composed largely of mineral crystals of nearly identical composition, the question arose as to whether the physical properties, *viz.* density, control the adsorption of phosphate.

In prior studies, the powdered hard tissues were exposed to various solutions for relatively short periods (30 to 60 minutes); the periods were chosen experimentally by following the rate of adsorption and selecting a time at which a "plateau" of adsorption apparently was reached. An obvious extension of these tests was to expose the three tissues to solutions for longer periods of time and to observe the relative amounts of phosphate adsorbed by the three tissues. Surprisingly, after prolonged exposure there is a reversal of the order in which these tissues adsorb phosphate, so that enamel adsorbs more than dentin and dentin more than bone.

Procedure

Radioactive phosphate (P^{32}) solutions were prepared from red phosphorus bombarded in the cyclotron. The phosphorus was dissolved in aqua regia and neutralized with sodium carbonate to a pH of 7.0 (1). A 0.2 M aqueous solution of disodium phosphate was made up and radioactive phosphorus was then added so that 25 ml. contained a known radioactivity (about 10,000 counts per minute) as measured on the Geiger-Müller scale-of-four counter (3).

The tissue samples consisted of 50 mg. portions of powdered enamel, dentin, and bone, respectively, of uniform particle size. The enamel and

* This work was supported in part by grants from the Carnegie Corporation of New York and the Eastman Dental Dispensary of Rochester.

dentin were separated by the Manly-Hodge flotation method (4). The organic matter of the bone was extracted with 3 per cent KOH in ethylene glycol (5). Each sample (50 mg. of powdered tissue) was stirred with 25 ml. of radioactive phosphate solution at 40° for various lengths of time ranging from 1 to 64 hours. The tests were made up in quadruplicate except for the 64 hour experiment which was in duplicate. After stirring, the solutions were centrifuged for 10 minutes and decanted. The precipitates were washed twice with 20 ml. portions of distilled water for 10 minutes. After each washing they were again centrifuged and the fluid decanted. The precipitates were then dissolved in 2 ml. of 4 N HCl, diluted suitably with distilled water, and the radioactivity determined. Two counters were used during the experiment. In order to have comparable data, the phosphorus standards and potassium acetate standards were checked repeatedly on each counter.

TABLE I

Phosphorus Adsorbed by Various Calcium Phosphates from 0.2 M Solution of Sodium Phosphate

Time stirred	Average P picked up per gm. solid (X/M)		
	Bone	Dentin	Enamel
hrs.	*mg.*	*mg.*	*mg.*
1	61.5	30.2	10.6
2	65.5	38.9	26.8
4	71.7	48.2	42.3
8	75.8	57.1	44.3
16	77.1	68.2	58.7
32	87.1	68.1	91.6
64	102.2	122.0	136.4

The rate of rotation and size of the stirring propeller were suspected of having some effect on the rate of adsorption. A test of these variables indicated, however, that the size of the stirrer had no effect on the rate of adsorption and that an increase in the speed of stirring produced only a slight, probably insignificant, increase in the rate of adsorption. In this experiment 50 mg. bone samples were stirred with a 0.002 M solution of Na_2HPO_4 at 40° for 30 minutes. "Slow speed" indicates about one-half of the "fast speed" in the following tabulation of the mg. of P taken up per gm. of bone.

	Slow speed	Fast speed
Large stirrer	8.25	8.65
Small "	8.02	9.02

Results

Table I shows the amount of phosphorus adsorbed by bone, dentin, and enamel, respectively, when each was stirred with a 0.2 M sodium phosphate solution at 40° for periods of 1 to 64 hours. *Each tissue gave evidence of greater adsorption with longer time exposures to the solution.* In the earlier work (2) a rapid adsorption of marked phosphorus was observed up to 30 minutes exposure to the solutions and thereafter a semblance of a plateau was reached. From the data in Table I it is evident that the plateau at 45 to 60 minutes was an artifact and that a fairly rapid adsorp-

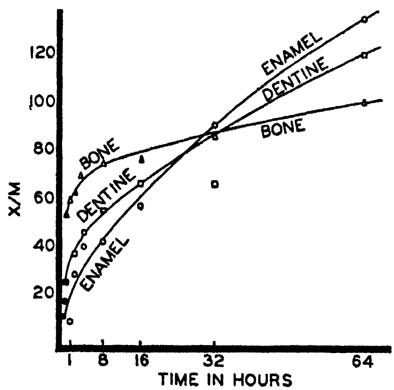


FIG. 1

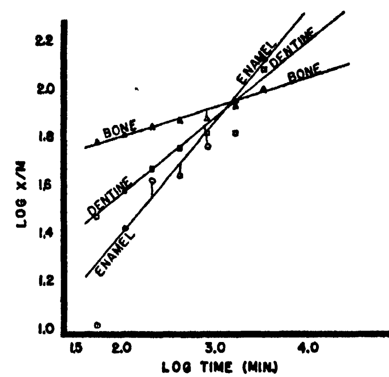


FIG. 2

FIG. 1. Adsorption of radioactive phosphorus by powdered bone, dentin, and enamel. The ordinate X/M represents mg. of phosphorus per gm. of tissue. The abscissa is the total time exposure of the powdered tissues to the radioactive Na_2HPO_4 solutions. Note the reversal of the order in which the three tissues adsorb phosphate.

FIG. 2. Amounts of radioactive phosphorus adsorbed by samples of bone, dentin, and enamel. The ordinate represents the logarithm of the mg. of phosphorus per gm. of hard tissue. The abscissa is the exposure time expressed in logarithms. In general, there is a straight line relationship between the concentration and the time. The slopes for the three tissues are in the order enamel > dentin > bone.

tion of marked phosphorus occurs in each sample over at least 8 hours exposure time.

After 1 hour the order of the amounts adsorbed was bone > dentin > enamel. The values quantitatively confirm those obtained previously (2), except that for enamel, which was lower in this 60 minute period than was found previously after 30 minutes. At 32 hours the order of the amount of adsorption was altered so that enamel > bone > dentin (Fig. 1). *Thus, at the longest exposure time, there is a complete reversal of the order of adsorption (1, 2) obtained with short exposure times.*

DISCUSSION

A number of comparable studies with radioactive isotopes as tracers have been carried out (2, 6, 7). In each test the three tissues studied have shown the same order of uptake; *viz.*, bone, dentin, enamel. This order seemed to have a plausible explanation based on the order of fundamental crystal size, which is just the reverse; the smaller the crystals the larger the effective adsorbing surface (8). Another explanation might be based on the different densities and porosities of the three tissues. Thus, it may be assumed that the phosphate solution can penetrate bone (density = 2.0) more rapidly than the dentin (density = 2.1 to 2.3) or the enamel (density = 2.9 to 3.0). More rapid penetration (bone > dentin > enamel)

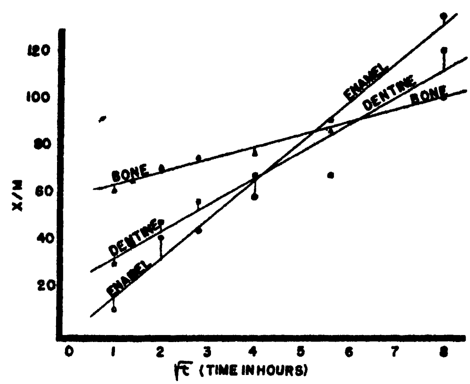


FIG. 3. Relation of the concentration of radioactive phosphorus to the square root of the exposure time. The ordinate X/M represents mg. of radioactive phosphorus per gm. of powdered tissue. The abscissa is the square root of the exposure time. In general, a straight line relationship is exhibited. The time constants for the three tissues are enamel 0.002, dentin 3.5, and bone 123.

would provide an explanation for the general rate relations shown in Fig. 1, and the high densities would make continuing adsorption plausible.

The order of adsorption after lengthy exposures, *viz.* enamel > dentin > bone, cannot be explained on the basis of the crystal sizes or differences in densities. The fundamental processes occurring after penetration are unknown; however, they seem to be in some way characteristic of each tissue. Thus, in Fig. 2, where the logarithms of the mg. of phosphate adsorbed are plotted against the logarithms of the exposure times, each tissue is found to exhibit a linear dependence of "turnover" on the logarithm of time. These curves show slopes for the three tissues in the order enamel > dentin > bone. What controls this long time tendency for enamel to exhibit greater quantitative adsorption is a difficult question. The basis may be in the

chemical differences in enamel, dentin, and bone cited by Armstrong and others (9, 10); the basis may be some physical characteristic; certainly at the present time the causes are hidden.

King, Cole, and Oppenheimer (11) have evolved an equation[1] describing certain time-concentration curves which they obtain in a study of the diffusion of the dye, T-1824, from blood plasma. When applied to our data for adsorption times of 1 to 64 hours, their equation gave a linear relationship between the "adsorption" of radioactive phosphate and the square root of time (Fig. 3), which indicates that diffusion is the limiting factor for each tissue.

At 64 hours, sufficient radioactive phosphate is present in the powdered tissues to require that a large proportion[2] of the total phosphate present must have exchanged (if exchange is the only mechanism by which marked phosphorus is taken up by the solid particles). Since equilibria are slowly attained in colloidal systems, it seems improbable that the values even after 64 hours of exposure represent any definable state. It is improbable that the uptake of marked phosphorus represents any preferential exchange of P^{32} from the solution for P^{31} from the solid.

SUMMARY

1. Powdered bone, dentin, and enamel adsorb marked phosphates from neutral solutions of sodium acid phosphate at 40°. Increasing amounts of marked phosphate are adsorbed by each of these tissues with increasing exposure times (at least up to 64 hours).

2. Up to 16 hours exposure time, the tissues adsorb phosphate in the following order, bone > dentin > enamel. This order is found to be reversed at 64 hours.

The authors wish to thank Dr. Robley D. Evans of the Massachusetts Institute of Technology for the radioactive phosphorus; Mr. J. F. Bonner for assistance in connection with the Geiger counters; and Dr. K. S. Cole,

[1] The equation mentioned above is $c = c_0(1 - \sqrt{t/T})$ where c is the concentration of radioactive phosphate at time t, c_0 is a concentration constant, and T is the time constant of the process. The time constant appears to be some function of the diffusion constant. The regression lines given in Fig. 3 were calculated from

$$\bar{c} - \frac{g}{h}\overline{\sqrt{t}}\left(1 - \frac{\sqrt{t}}{\overline{\sqrt{t}} - \bar{c}\dfrac{g}{h}}\right)$$

where $g = \Sigma(\sqrt{t}c) - \overline{\sqrt{t}}\Sigma c$ and $h = \Sigma t - \overline{\sqrt{t}} \cdot \Sigma\sqrt{t}$.

[2] The proportion of total phosphate which must have been exchanged was calculated to be 72 per cent for bone, 81 per cent for dentin, and 82 per cent for enamel.

Dr. R. W. Brauer, and Mr. J. A. Rafferty for discussions of the mechanism of the phenomena described. The authors acknowledge the assistance of Miss Elizabeth Street with the statistical computations.

BIBLIOGRAPHY

1. Manly, M. L., and Levy, S. R., *J. Am. Chem. Soc.*, **61**, 2588 (1939).
2. Hodge, H. C., Van Huysen, G., Bonner, J. F., and Van Voorhis, S. N., *J. Biol. Chem.*, **138**, 451 (1941).
3. Bale, W. F., Haven, F. L., and LeFevre, M., *Rev. Scient. Instruments*, **10**, 193 (1939).
4. Manly, M. L., and Hodge, H. C., *J. Dent. Res.*, **18**, 133 (1939).
5. Crowell, C. D., Hodge, H. C., and Line, W. R., *J. Dent. Res.*, **14**, 251 (1934).
6. Volker, J. F., Hodge, H. C., Wilson, H. J., and Van Voorhis, S. N., *J. Biol. Chem.*, **134**, 543 (1940).
7. Hodge, H. C., Koss, W. F., Ginn, J. T., Falkenheim, M., Gavett, E., Fowler, R. C., Thomas, I., Bonner, J. F., and Dessauer, G., *J. Biol. Chem.*, **148**, 321 (1943).
8. Bale, W. F., Hodge, H. C., and Warren, S. L., *Am. J. Roentgenol.*, **32**, 369 (1934).
9. Armstrong, W. D., and Brekhus, P. J., *J. Biol. Chem.*, **120**, 677 (1937).
10. Armstrong, W. D., in Annual review of biochemistry, Stanford University, **11**, 441 (1942).
11. King, B. G., Cole, K. S., and Oppenheimer, E., *Am. J. Physiol.*, **138**, 636 (1942).

THE LIPIDS OF THE PIG DURING EMBRYONIC DEVELOPMENT

By WILLIS A. GORTNER*

(From the Department of Biochemistry and Pharmacology, School of Medicine and Dentistry, The University of Rochester, Rochester, New York)

(Received for publication, March 21, 1945)

The bulk of the literature on embryochemical growth has been devoted to changes in the embryonic chick and fish. Unfortunately the unique position of the mammalian fetus, the only one not developing in a closed system, prevents any generalization of conclusions drawn from observations on the eggs of these lower forms of animals. Parallel growth changes are infrequent even between different mammalian species, since the placenta in one may offer a set of barriers to the passage of a particle which freely crosses the placental membranes in another species of mammal. The human placenta, for example, interposes three barriers between the fetal and maternal bloods, while in the pig no less than six membranes are interposed between the two circulatory systems.

The only paper approaching completeness in dealing with the evolution of the various lipids in the mammalian fetus is that in which Boyd (5) studied the rôle of the placenta in the transfer of lipids to the rabbit fetus. For the purpose of the present study the pig fetus was selected as offering unique possibilities for an analysis of lipid development. Since the pig placenta offers a maximum number of barriers to the passage of particles from the maternal blood to the fetus, one might expect the fetal pig to exhibit a greater ability to synthesize its tissue components than is the case with other mammals, such as man and the rabbit. The complexity of the placenta in the pig, together with the pooling of fetuses from different litters for the various analyses, should reduce the effect of dietary variations in the mother.

Methods of Analysis

The pig has a gestation period of from 110 to 120 days, or approximately 4 months (17, 20). During the first 3 weeks, the fetuses are so small (15 mm. or less) that it has not been possible to obtain sufficient numbers for analysis. The period of most rapid growth rate, *i.e.* the 2nd and 3rd months, has been divided into approximately 5 day periods, the fetuses falling within a given age range being pooled until a sufficient number had been obtained for analysis; older fetuses were classed into groups with a 10 day age range. The samples were thus assigned to one of the following

* Present address, School of Nutrition, Cornell University, Ithaca, New York.

groups (the figures representing the crown-rump length of the fetus in mm.): 16 to 30, 31 to 50, 51 to 60, 61 to 80, 81 to 100, 101 to 110, 111 to 120, 121 to 140, 141 to 160, 161 to 175, 176 to 190, 191 to 200, 201 to 230, 231 to 260. Since the length of the pig fetus has been correlated with the intrauterine age by various workers (17, 20, 21, 23), it appears safe to conclude that fetuses of the same length are in the same phase of embryonic development.

The specimens were collected within a half hour of the death of the mother, and usually showed life as exhibited by the fetal heart beat. The uterus was opened, the fetus dissected free from attaching membranes, dried with filter paper or gauze pads, and the crown-rump length recorded. After an immediate weighing, the abdominal and cranial cavities were opened to insure access of solvent to the tissues, and the specimens were placed in 95 per cent ethyl alcohol for dehydration and extraction. The collections were brought to the laboratory, ground to a fine pulp in a food chopper, care being taken to rinse all particles back into the flask with additional alcohol, and allowed to stand at room temperature for a minimum of 24 hours in an amount of alcohol at least equal to that of the volume of pulped tissue.

The tissue was then exhaustively extracted with alcohol and ether and the combined extracts concentrated *in vacuo* at 40° to a small volume. This concentrate was in turn repeatedly extracted with redistilled petroleum ether. The clear petroleum ether solution, containing only the lipids, was made up to a suitable volume in a volumetric flask, from which aliquots were taken for the various analyses.

The water-alcohol layer remaining after extraction with petroleum ether was evaporated to dryness and the caramel-like residue weighed. This "tar," containing proteins, salts, carbohydrates, etc., soluble in the earlier, more watery extracts, was present in sufficient amounts to necessitate including it in calculating the "dry weight" of the fetuses.

The extracted tissue was weighed after removal of the remaining solvent. This weight, plus the sum of the weights of the "tar" and the lipid, gave the "dry weight" of the sample from which the per cent of water in the fetuses was calculated.

Most of the various lipid analyses were run in triplicate or quadruplicate (occasional determinations were run in duplicate only). *Total lipid* was determined gravimetrically on approximately 50 mg. samples. *Free fatty acids* were determined on these lipid samples by adding 10 ml. of hot, neutral 95 per cent alcohol and titrating with standard 0.05 N NaOH to an α-naphtholphthalein end-point. The calculations for free fatty acids were made on the assumption (upheld by iodine number and molecular weight determinations) of a mean molecular weight equal to that of oleic acid (mol. wt. 282.3).

Unsaponifiable lipids were determined gravimetrically on 50 mg. samples according to the procedure recommended by Kelsey (16).

The *total* and *free cholesterol* fractions were determined by the method of Kelsey (15).

Phospholipids were determined by three methods, each focusing itself on either a different part of the phospholipid molecule, *viz.* the phosphorus (18) or the fatty acids (2), or on the intact molecule (3). The comparative data for the three methods have been previously reported (9). The averages of the values obtained by the three methods have been used in the present paper.

Iodine numbers were determined on the total and phospholipid fatty acids by the method of Yasuda (25).

DISCUSSION

The results of the analysis of twenty-six groups of pig fetuses, representing 438 fetuses from 66 litters, and ranging from 17 to 254 mm. in length, are presented in Tables I and II.

Water—The water content of the fetuses at different developmental stages shows a decided downward trend throughout the gestation period, the most rapid changes occurring before the 50 mm. and after the 200 mm. stages. Wilkerson and Gortner (24) have correlated the changes in the water content of the pig fetus with anatomical differentiation in the fetal kidneys. These authors secured data indicating a rapid fall in water content at the 15 mm. stage, at which level it remained constant until the fetus reached a length of 160 mm. The values reported here show a more gradual initial fall, and give no indication of an equilibrium in water content between the 15 and 160 mm. stages; instead, they show a small but steady drop in the per cent of water during this period.

Total Lipid—The lipid fraction remains constant during a considerable part of the gestation period. Calculated in terms of the entire fetal weight, the lipid content remains at slightly over 1 per cent until the last month of fetal life, at which time it rises slightly to a value of 1.4 per cent near term. This behavior of the lipid is in striking contrast to that observed in other mammals. The fetuses of rabbits (5, 8), dogs (19), and man (8, 14) exhibit a sharp rise of 300 to 800 per cent in the lipid content during the latter part of the gestation period.

Of interest is the close parallelism of the nitrogen and lipid contents of the pig fetus. Wilkerson and Gortner (24) found the protein nitrogen to be constant after the 50 mm. stage; on a dry weight basis, total lipids remain at a constant level after the 60 mm. stage, indicative of a fairly rigid ratio of these substances in developing tissues.

Free Fatty Acids—The data for free fatty acids in developing pig fetuses as presented in Table II indicate that unesterified fatty acids are present

in considerable amounts, especially in the earlier periods of prenatal life. The very definite trend in the free fatty acid content during embryonic development, together with the precautions followed to avoid autolysis,

TABLE I

Changes in Size and Water Content of Pig during Embryonic Development

Average length of fetus	Estimated conception age of fetus	No. of fetuses	No. of litters	Average weight of fetus	Water content
mm.	*days*			*gm.*	*per cent*
17	25	71	8	0.38	92.6
21	27	30	4	0.71	91.2
22	28	35	4	0.92	91.2
23	28	50	7	0.88	91.3
28	32	18	3	1.91	91.4
29	32	52	8	2.07	91.0
34	34	13	1	3.19	90.8
38	36	38	6	4.44	91.2
39	37	33	4	5.08	90.7
55*	42	2	1	11.2	90.1
56	42	20	2	11.0	89.8
65*	45	3	1	13.9	90.0
66	45	6	1	16.0	89.7
72	47	14	2	22.2	89.7
85	51	5	1	30.2	89.0
95	55	8	1	41.5	89.8
103	57	5	1	54.4	89.5
105	58	6	2	64.4	88.8
107	59	8	1	60.8	89.5
114	61	6	1	65.1	88.5
118	62	3	1	80.2	89.2
135	68	5	2	114.7	88.8
187	84	2	2	302.0	88.4
189	85	2	1	321.2	88.4
196	87	2	1	402.0	88.5
254	105	1	1	779.0	85.3

* Samples from the same litter.

minimize the possibility that these acids are artifacts arising from the decomposition of some other lipid.

The presence of considerable amounts of fatty acids in unesterified form in the very small fetuses[1] suggests the possibility that certain enzymes asso-

[1] It is interesting to note the similarity in trends for free fatty acids and for basic nitrogen in terms of tissue solids. The basic nitrogen falls rapidly until the 40 to 50 mm. stage, after which it remains relatively constant (24). It is impossible to say whether the similar behavior of these two groups of compounds is other than coincidental. That the free fatty acids are in some way associated with the basic nitrogenous substances, however, seems probable. The existence of free fatty acids as

ciated with lipid metabolism are absent (or of very low potency) in the young fetuses, and are formed (or assume function) at about the 25 mm. stage.

TABLE II

Lipids of Pig at Various Stages of Embryonic Development

The concentrations of the lipid fractions are expressed as per cent of the dry weight of the fetus.

Average length of fetus	Total lipid	Free fatty acids	Phospholipid*	Unsaponifiable lipids	Cholesterol		Glycerides†	Iodine No.‡	
					Total	Free		Total fatty acids	Phospholipid fatty acids
mm.									
17	15.9	2.42	8.12	2.95	1.67	1.45	2.2	96	86
21	15.7	2.36	7.80		2.14	2.07		113	79
22	10.8	1.25	6.50	2.63	1.77	1.46	0.3	77	87
23	13.2	1.60	7.68	3.12	1.85	1.46	0.5	77	68
28	11.1	1.98	6.72	2.35	1.72	1.57	0.0	85	82
29	11.3	1.10	7.36	2.15	1.60	1.32	0.5	84	83
34	11.6	1.35	7.21	2.60	1.54	1.17	0.2	72	71
38	12.4	1.16	7.61	2.24	1.41	1.33	1.3	110	97
39	11.5	1.27	7.35	2.35	1.55	1.34	0.4	84	69
55§	10.7	1.18	6.33	2.37	1.40	1.21	0.7	57	42
56	10.8	1.07	7.05	2.22	1.48	1.29	0.3	76	71
65§	8.9	1.16	5.47	2.11	1.49	1.36	0.0	76	76
66	11.1	1.29	6.68	2.29	1.26	1.23	0.8	77	95
72	9.2	0.92	5.86	1.80	1.30	1.08	0.5	83	93
85	9.3	0.98	5.68	1.97	1.22	1.18	0.7	87	114
95	10.1	1.02	6.16	1.83	1.19	0.94	0.9	74	59
103	9.1	0.95	5.85	1.68	1.22	1.00	0.5	73	71
105	9.5	1.08	5.39	1.79	1.20	1.08	1.1	78	102
107	10.4	1.33	6.51	1.86	1.33	1.10	0.5	74	85
114	9.0	1.02	5.18	1.56	1.11	1.05	1.2	60	64
118	8.7	1.06	5.03	1.83	1.12	1.03	0.7	68	78
135	9.6	0.77	5.41	1.65	1.20	1.00	1.7	97	93
187	9.6	0.77	4.90	1.70	1.16	0.96	2.1	75	76
189	9.1	0.78	4.67	1.61	1.21	0.97	1.9	79	86
196	8.8	0.68	4.25	1.52	1.05	1.03	2.3	124	115
254	9.5	0.65	4.08	1.37	1.05	0.93	3.3	89	82

* Average of three methods of determination (*cf.* (9)).

† By difference.

‡ Based on oxidation values for the fatty acid solutions.

§ Samples from the same litter.

Phospholipids—The average values for phospholipids, when calculated as per cent of the wet weight of the fetus, show little change at any time

salt complexes with basic amino acids seems more logical than the presence of uncombined fatty acids as such in the tissues.

during the gestation period. This is quite different from the trend in the fetal rabbit (5), in which the concentration of phospholipids increased throughout pregnancy, a 5-fold increase being effected during the period studied. Calculated on either a wet or dry weight basis, the phospholipids of the fetal pig are at a maximum during the early stages of intrauterine development.

Unsaponifiable Lipids—The total unsaponifiable matter, as determined gravimetrically, was found to contain variable amounts of digitonin-precipitable sterols (55 to 76 per cent, calculated as cholesterol). In general the trend is toward a higher ratio of sterol to non-sterol unsaponifiable lipids as the fetus becomes more developed.

Expressed as per cent of the dry weight of the fetus, the values for unsaponifiable lipid decrease during the developmental period to less than half the original value, falling on a smooth curve which shows the change to be more rapid during the early stages of embryonic growth.

The cholesterol, as one of the constituents of the unsaponifiable material, exhibits a similar trend. The *total* and *free cholesterol* contents parallel, at lower levels, the changes in unsaponifiable matter. The *bound cholesterol*, while distributing itself over a fairly wide range of values, shows no change during fetal growth. While the latter is entirely in agreement with the findings of Boyd (5) on rabbit fetuses, the trend for free cholesterol is quite different. In the pig, little change is evident in the free cholesterol content of the moist fetal tissues during intrauterine development. In the rabbit fetus, however, the free cholesterol doubles in concentration during embryonic growth, increasing particularly in the early and in the very late stages. This again emphasizes the difficulty of generalizing in interpreting data on fetal composition without due regard to the type of placenta linking the fetus to the mother.

At no time does there appear to be any appreciable demand or tendency for cholesterol to appear in ester form, an average of only 1 out of every 8 cholesterol molecules being in the bound fraction. In general, only small amounts of cholesterol esters are found in active tissues, the content varying inversely with the physiological activity of the organ (4, 12, 22). The low ester cholesterol values[2] in the actively growing fetuses are in harmony with these findings.

Neutral Fat—"Glycerides" were determined by difference, being that portion of the total lipid not accounted for as phospholipid, free fatty acid, unsaponifiable lipid substance, or cholesterol ester fatty acid.[3] Since

[2] Hanes (10) notes that, while the liver of the fetal dog contains large amounts of "anisotropic lipoid substance" (cholesterol esters), he was unable to find such substances in the liver of the pig fetus in any of the stages of development.

[3] Fatty acids combined as cholesterol esters were assumed to have a molecular weight of 283.

certain assumptions as well as analytical errors are necessarily made in calculating the amounts in each of these four fractions, the data for the glycerides cannot be considered other than as relative values. The scattering of the data is undoubtedly greater than the true fluctuations of the neutral fat; the trend, however, is readily apparent.

The glycerides remain at a very low level until approximately the middle of the gestation period. After the 100 mm. stage is reached, the glycerides increase steadily. This increase in neutral fat during the last half of the gestation period is attained more gradually than the huge increase found in the fetal rabbit (5), and the absolute values are far lower in the pig, which shows less than half as much infiltrated fat as is found in the rabbit near term. In the rabbit, the curve for neutral fat has leveled off before parturition; in the pig, the peak has not been attained at term. This is confirmation of the observation of Engel and Bode (7) that pig fetuses have a minimum enrichment of fat stores before parturition, in contrast to the full depots of the rabbit (5) and man (8).

Iodine Numbers of Fatty Acids—In Table II are listed the values for iodine numbers of the total and phospholipid fatty acids. The majority of the values for both fractions lie between an iodine number of 60 and 110, the average value for both the total and the phospholipid fatty acids being 82. In neither case is there any indication of a shift toward either a greater or lesser degree of unsaturation during progressive stages of fetal development, despite the fact that the total fatty acid fraction is made up of 70 per cent phospholipid and 25 per cent free fatty acids at one stage, and 45 per cent phospholipid and 45 per cent glyceride fatty acids at another stage of embryonic growth. The fatty acids have an average unsaturation equivalent to one double bond; the average analyses (iodine number 82 $\pm$ 3.1, molecular weight 283 $\pm$ 2.7[4]) compare closely with the values for oleic acid (90 and 282, respectively).

The iodine numbers of the fatty acids in the fetus differ appreciably from those found in adult tissue. In general the fatty acids bound to phospholipids in adult tissue are considerably less saturated than those on the glyceride molecules; the phospholipid fatty acids of the fetal pig have an iodine number similar to the fatty acids on the other lipid components. In the present study, an average iodine number of over 80 for the glyceride fatty acids is indicated, a value well above the reported average of 63 for the back fat of the sow (1, 13), which is representative of the entire body fat of the pig (6). This is in line with the observation of Hankins and Ellis (11) that the body fat of immature hogs is usually soft, becoming firmer as they approach maturity.

[4] Mean ($\pm$ standard error) of triplicate titrations of the fatty acids of ten lipid samples.

SUMMARY

The lipid fractions of 438 fetuses, representing 66 litters and covering 70 per cent of the gestation period of the pig, have been investigated.

The water content of the pig fetus exhibits two rapid falls during growth, a phenomenon previously correlated with changes in the fetal kidneys.

The total lipid and the lipid to protein ratio remain constant for a large part of the embryonic growth period.

Evidence is presented that a considerable portion of the non-phospholipid fatty acids, often considered "neutral fat," is actually present in unesterified (free) form.

On a dry weight basis, the phospholipid content is at a maximum in the very young fetus, which has twice as much of this lipid as does the fetus at term. The phospholipid fatty acids, in common with the other acid fractions, have an average iodine number of 82.

The unsaponifiable lipids in the dry solids progressively decrease in their percentage content during embryonic growth, the total and free cholesterol fractions roughly paralleling this fall. At no time is there any notable tendency for cholesterol to appear in ester form.

The fetal glycerides gradually increase beginning about the middle of the gestation period, but even at term they account for only a minor part of the total lipid substance.

Considerable differences exist in the development of the lipids in the fetal pig in comparison with the fetal rabbit.

BIBLIOGRAPHY

1. Banks, A., and Hilditch, T., *Biochem. J.*, **26**, 298 (1932).
2. Bloor, W. R., *J. Biol. Chem.*, **80**, 443 (1928).
3. Bloor, W. R., *J. Biol. Chem.*, **82**, 273 (1929).
4. Bloor, W. R., Okey, R., and Corner, G. W., *J. Biol. Chem.*, **86**, 291 (1930).
5. Boyd, E. M., *Biochem. J.*, **29**, 985 (1935).
6. Ellis, N. R., and Isbell, H. S., *J. Biol. Chem.*, **69**, 219 (1928).
7. Engel and Bode, *Monatschr. Kinderh.*, **8**, 618 (1909).
8. Fehling, H., *Arch. Gynäk.*, **11**, 523 (1877).
9. Gortner, W. A., *J. Biol. Chem.*, **159**, 97 (1945).
10. Hanes, F. M., *J. Exp. Med.*, **16**, 512 (1912).
11. Hankins, O. G., and Ellis, N. R., *U. S. Dept. Agr., Bull. 1407* (1926).
12. Haven, F. L., *Am. J. Cancer*, **29**, 57 (1937).
13. Hilditch, T., and Stainsby, W., *Biochem. J.*, **29**, 90 (1935).
14. Iob, V., and Swanson, W., *Am. J. Dis. Child.*, **47**, 302 (1934).
15. Kelsey, F. E., *J. Biol. Chem.*, **127**, 15 (1939).
16. Kelsey, F. E., *J. Biol. Chem.*, **130**, 187 (1939).
17. Koch, M. L., *J. Biol. Chem.*, **14**, 267 (1913).
18. Kuttner, T., and Lichtenstein, L., *J. Biol. Chem.*, **86**, 671 (1930).
19. Liesenfeld, F., Dahmen, H., and Junkersdorf, P., *Arch. ges. Physiol.*, **216**, 712 (1927).

20. Mendel, L. B., and Mitchell, P. H., *Am. J. Physiol.*, **20**, 81 (1907).
21. Mendel, L. B., and Leavenworth, C., *Am. J. Physiol.*, **20**, 117 (1907).
22. Okey, R., Bloor, W. R., and Corner, G. W., *J. Biol. Chem.*, **86**, 307 (1930)
23. Warwick, B. L., *J. Morphol. and Physiol.*, **46**, 59 (1928).
24. Wilkerson, V., and Gortner, R. A., *Am. J. Physiol.*, **102**, 153 (1932).
25. Yasuda, M., *J. Biol. Chem.*, **94**, 401 (1931–32).

THE UTILIZATION OF CHOLINE ANALOGUES BY CHOLINELESS MUTANTS OF NEUROSPORA

By N. H. HOROWITZ, DAVID BONNER, AND MARY B. HOULAHAN

(From the School of Biological Sciences, Stanford University, California)

(Received for publication, April 2, 1945)

In a previous report (1) a choline-requiring mutant of *Neurospora crassa* has been described. It was shown that the mutant fails to grow on an unsupplemented basal medium but grows normally (*i.e.*, at the wild type rate) when choline is supplied. Lecithin is also active in promoting growth; the slight activity of methionine was interpreted as a sparing action on choline. A number of other compounds, structurally related to choline, including betaine and aminoethanol, were found to be inactive. In a note recently published Jukes and Dornbush (2) have shown that dimethylaminoethanol is active for this mutant, in agreement with the results reported below.

Recently a new *cholineless* strain differing genetically from the original one was obtained from a culture of wild type *Neurospora crassa* irradiated with ultraviolet light. On the basis of the conclusion drawn from previous studies that a one-to-one relation exists between genes and biochemical reactions (3), it is to be expected that these mutants differ biochemically as well as genetically. An examination of the structural specificity of choline in promoting the growth of the two mutants was therefore undertaken as part of a general investigation into the biochemistry of the strains.

EXPERIMENTAL

Mutant Strains—Four independent occurrences of the *cholineless* character have been obtained from irradiated *Neurospora; viz.*, strains 34486, 34542, 37903, and 47904. Genetic tests which show that strain 34486 differs from the wild type by a single gene have been reported (1). The results of outcrosses involving the other three mutants indicate that in these strains also the *cholineless* character is associated with a single gene change. However, the results obtained by crossing the four mutant types among themselves show that strains 34486, 34542, and 37903 carry mutations of the same gene, while the mutation in strain 47904 affects a different gene. All asci (twenty from each cross) obtained from crosses of strain 34542 to strains 34486 and 37903 contained only *cholineless* spores, indicating that these genes are probably allelic. On the other hand, crosses of strain 47904 to strains 34486 and 34542 gave rise to asci containing both wild type and double mutant spores. The double mutant nature of the

latter was established by recovering the original monogenic strains from outcrosses of the double mutants to the wild type. These results prove that two different *cholineless* genes entered each cross.

The experiments reported in this paper were carried out with strains 34486 and 47904. The former is designated *cholineless-1* and the latter *cholineless-2*.

Compounds—We are indebted to Dr. H. J. Almquist for a sample of arsenocholine chloride originally prepared by Dr. A. D. Welch, to Dr. A. D. Welch for a sample of calcium phosphorylcholine chloride, and to Dr. Vincent du Vigneaud for samples of triethylcholine chloride and dimethylethylhydroxyethylammonium chloride.

Neurine bromide was prepared by the treatment of choline chloride with bromine to give bromocholine bromide (4), and this compound was in turn converted to neurine bromide according to the method of Renshaw and Ware (5).

Monomethylaminoethanol was prepared from ethylene oxide and aqueous methylamine according to the method of Knorr and Matthes (6).

Dimethylaminoethanol was prepared by adding ethylene oxide to 25 per cent aqueous dimethylamine in the cold as described by Knorr and Matthes (7). The resulting mixture was fractionally distilled, the fraction boiling between 130–140° being retained. This fraction is principally dimethylaminoethanol but does contain approximately 2 per cent water (7). The chloroaurate was prepared and had a melting point of 197°, in agreement with that given by Knorr (8). Analysis showed the following.

$C_4H_{11}NO\cdot HCl\cdot AuCl_3$.	Calculated.	C 11.18, H 2.79, Au 45.97
	Found.	" 11.17, " 2.81, " 46.04

Diethylmethylhydroxyethylammonium chloride was prepared by condensing diethylaminoethanol with methyl iodide (9) in dry ether in the cold. Diethylmethylhydroxyethylammonium iodide precipitated overnight in the refrigerator and was purified by dissolving in ethanol and crystallizing by the addition of ether. The iodide was replaced with chloride by treatment with silver chloride. Analysis of the crystalline reineckate gave the following.

$C_7H_{18}NO\cdot Cr(NH_3)_2(SCN)_4$.	Calculated.	C 29.33, H 5.33, N 21.78
	Found.	" 29.15, " 5.30, " 21.84

Growth Studies—The results found by growing the mutants in liquid culture are summarized in Table I. In these experiments the dry weight attained by the mold grown in the presence of various concentrations of each test substance was measured. The cultures were incubated at 25° for 72 hours in the medium previously described (1). A curve relating growth rate to concentration was obtained for each analogue, but only

optimum concentrations are shown here, together with the maximum growth rate attained on each compound.

It was found that *cholineless-2* responds to much lower concentrations of most of the active compounds tested than does *cholineless-1*. The concentration of choline required to produce normal growth of the former strain is only 0.1 to 0.2 that required by the latter. The explanation of this difference appears to rest on the fact that *cholineless-2* is not completely deficient in the ability to synthesize choline when grown in liquid culture. In contrast to *cholineless-1*, which produces only negligible growth in unsupplemented media, *cholineless-2* produces 5 to 15 mg. of dry weight in 72 hours, or up to 20 per cent of the dry weight attained on an optimum concentration of choline. When incubated for longer than 72 hours, *cholineless-2* continues to grow at a subnormal rate in the unsupplemented medium until the nutrients are exhausted. It seems clear from this finding that, although the choline-synthesizing mechanism is seriously impaired in this mutant, it is not entirely lacking. As a result, the requirement of this strain for exogenous supplies of choline or choline analogue is not so great as is that of *cholineless-1*, in which choline synthesis is completely blocked.

In the light of the above findings the difference in the ability of the two mutants to utilize monomethylaminoethanol is especially noteworthy. *Cholineless-2* shows a marked deficiency in the ability to utilize this analogue. Compared with *cholineless-1* its requirement is higher, and the maximum growth rate attained is considerably less than normal.

In the course of the genetic investigation of the mutants it was observed that, except for an initial germination stage, *cholineless-2* fails to grow on an unsupplemented agar medium, even after 7 days incubation. This is in marked contrast to the slow, but continuous, growth produced in liquid culture. The explanation of this behavior is not known. However, several other instances are known in which the ability of *Neurospora* mutants to synthesize specific growth factors depends on the culture conditions. Published examples are the pH-dependent *pyridoxineless* strain 299 (10) and *arginineless* strain 33442 (11) and the temperature-dependent *adenineless* strain 44206 (3).

In view of the above finding it was of interest to retest the analogues on a solid medium. This was done by means of the Petri plate method described by Thompson, Isbell, and Mitchell (12). Petri plates containing the same basal medium as is used in liquid culture plus 2.5 per cent agar were inoculated with agar plugs containing a suspension of germinated spores. The rate of linear growth of the mold over the agar plate was determined by measuring the diameter of the circle formed by the growing mycelium after 18 hours at 25°. In accordance with a suggestion by

Dr. H. K. Mitchell, the original procedure was modified by omitting the autoclaving of the agar plates prior to inoculation. The growth of *Neurospora* is so rapid and the incubation time is so short that no interference from contaminating organisms need be feared.

TABLE I

Activity of Various Compounds in Promoting Growth of Cholineless Mutants in Liquid Culture

Compound	Optimum concentration*				Maximum response†	
	Strain 34486		Strain 47904		Strain 34486	Strain 47904
	γ per ml.	*moles per ml. × 10⁹*	*γ per ml.*	*moles per ml. × 10⁹*	*per cent*	*per cent*
Choline	1.0	8.25	0.2	1.65	100	100
Acetylcholine chloride	6.0	33.0	3.0	16.5	100	100
Arsenocholine "	1.65	8.25	0.44	2.20	100	100
Ca phosphorylcholine chloride	8.0	31.0	3.0	11.6	100	100
Dimethylaminoethanol	1.2	13.4	1.2	13.4	100	100
Monomethylaminoethanol	4.0	53.2	10	133	100	75
Dimethylethylhydroxyethylammonium chloride	4.0	26.0	2.0	13.0	100	100
Diethylmethylhydroxyethylammonium chloride	120	716	80	477	100	90
Triethylcholine chloride	240	1320	80	440	61	73
dl-Methionine	60	402	6.0	40.2	25	79
Betaine					0	0
Creatine					0	0
Ethanolamine					0	0
Sarcosine					0	0
Neurine bromide					0	0
Diethylaminoethanol					0	0
Dimethylamine					0	0
Trimethylamine					0	0
Tetramethylammonium chloride					0	0

* Optimum concentration is the lowest concentration of the test substance above which little or no further increase of growth rate is obtained.

† Maximum response is the growth rate attained at the optimum concentration, expressed as a percentage of that attained on choline.

The results are summarized in Table II. When grown on agar, *cholineless-2* is found to require more choline for maximum growth than *cholineless-1*. This is referable to the difference of growth habit of the strains on solid medium. The mat formed by *cholineless-2* is denser, with considerably more aerial growth, than that formed by *cholineless-1*. Consequently, for equal surface areas covered *cholineless-2* produces more mass

than does *cholineless-1*, and its apparent choline requirement is therefore higher.

In agreement with the findings obtained in liquid culture, the mutants show a significant difference in their ability to utilize monomethylaminoethanol when cultured on solid medium. In addition, *cholineless-2* shows

TABLE II

Activity of Various Compounds in Promoting Growth of Cholineless Mutants on Solid Medium

Compound	Optimum concentration				Maximum response	
	Strain 34486		Strain 47904		Strain 34486	Strain 47904
	γ per ml.	*moles per ml. × 10⁹*	*γ per ml.*	*moles per ml. × 10⁹*	*per cent*	*per cent*
Choline	0.4	3.30	1.2	9.90	100	100
Acetylcholine chloride	2.0	11.0	4.0	22.0	97	82
Arsenocholine "	0.8	4.00	2.0	10.0	91	97
Ca phosphorylcholine chloride	80	310	60	233	84	89
Dimethylaminoethanol	0.8	8.96	1.6	17.9	99	61
Monomethylaminoethanol	6.0	79.8	20	266	93	50
Dimethylethylhydroxyethylammonium chloride	3.0	19.5	8.0	52.0	88	100
Diethylmethylhydroxyethylammonium chloride	100	597	160	955	85	82
Triethylcholine chloride	>200*	>1100	>200	>1100	>80	>47
dl-Methionine	12	80.5	20	134	32	54
Betaine					0	0
Creatine					0	0
Ethanolamine					0	0
Sarcosine					0	0
Neurine bromide					0	0
Diethylaminoethanol					0	0
Dimethylamine					0	0
Trimethylamine					0	0
Tetramethylammonium chloride					0	0

* Highest concentration tested.

a deficiency in the utilization of dimethylaminoethanol. On neither compound does this mutant attain the normal growth rate, although *cholineless-1* does. If the utilization of dimethylaminoethanol involves its conversion to choline by the addition of a methyl group (13, 14), the results suggest that the cultural conditions (liquid *versus* solid) affect in some way the ability of *cholineless-2* to bring about this methylation.

DISCUSSION

The present experiments show that an inherent difference exists between the two *cholineless* mutants of *Neurospora* in their ability to utilize monomethylaminoethanol and dimethylaminoethanol. The results suggest that the gene-controlled deficiency in *cholineless-2* may be concerned with the methylation of a mono- or dimethylated precursor of choline, while that in *cholineless-1* blocks a prior step in the synthesis. The inability of *cholineless-2* to utilize mono- and dimethylaminoethanol is relative, not absolute as might be expected on the above interpretation. It is possible, however, that these compounds have a certain amount of intrinsic choline-like

TABLE III

Activity of Choline and Some of Its Analogues for Rat, Chick, and Neurospora

Unless otherwise indicated, data for the rat and chick are taken from the summary of Moyer and du Vigneaud (9). +, active; −, inactive; ±, less than 10 per cent as active as choline. The figures in parentheses represent bibliographic reference numbers.

Compound	Rat		Chick		*Neurospora*
	Growth on homocystine diet	Lipotropic	Growth	Perosis prevention	Growth
Choline	+	+	+	+	+
Arsenocholine	−	+	+	+	+
Dimethylaminoethanol	− (13)		+ (14)	+ (14)	+
Monomethylaminoethanol	− (13)				+
Dimethylethylhydroxyethylammonium chloride	+	+	+	+	+
Diethylmethylhydroxyethylammonium chloride	−	+	−	+	±
Triethylcholine	−	+	−	−	±
Betaine	+	+	±	−	−
Methionine		+	−	−	±

activity for *Neurospora*. It is of interest to note that the addition of ethyl groups to mono- and dimethylaminoethanol, giving the diethylmethyl and dimethylethyl analogues of choline, respectively, lowers the activity in terms of the optimum concentration for the mutants, but increases the activity in terms of maximum response by *cholineless-2*.

In studies now in progress it has been found that during growth in liquid *cholineless-2* accumulates an unknown substance which is active for *cholineless-1* and, to a lesser extent, for itself. Growth curves obtained with choline-free concentrates of the factor resemble those given by monomethylaminoethanol. Preliminary tests indicate that it is not identical with this

substance, however. Further characterization of the factor will have to await its isolation.

A number of interesting comparisons can be made between the activity of various analogues for the rat, chick, and *Neurospora*. These are shown for a number of selected compounds in Table III.

This work was supported by a grant from the Rockefeller Foundation.

SUMMARY

The response of two choline-requiring mutants of *Neurospora* to some analogues of choline was measured. The following compounds show some activity for both mutants: choline, acetylcholine, arsenocholine, phosphorylcholine, dimethylaminoethanol, monomethylaminoethanol, dimethylethylhydroxyethylammonium chloride, diethylmethylhydroxyethylammonium chloride, triethylcholine, and methionine. The following compounds are inactive for both mutants: betaine, creatine, sarcosine, ethanolamine, neurine, diethylaminoethanol, dimethylamine, trimethylamine, and tetramethylammonium chloride. The mutants show significant differences in their ability to utilize mono- and dimethylaminoethanol. The implications of this finding for the possible nature of the gene-controlled reactions is discussed.

BIBLIOGRAPHY

1. Horowitz, N. H., and Beadle, G. W., *J. Biol. Chem.*, **150**, 325 (1943).
2. Jukes, T. H., and Dornbush, A. C., *Proc. Soc. Exp. Biol. and Med.*, **58**, 142 (1945).
3. Horowitz, N. H., Bonner, D., Mitchell, H. K., Tatum, E. L., and Beadle, G. W., *Am. Naturalist*, in press (1945).
4. Schmidt, E., *Ann. Chem.*, **337**, 52 (1904).
5. Renshaw, R. R., and Ware, J. C., *J. Am. Chem. Soc.*, **47**, 2993 (1925).
6. Knorr, L., and Matthes, H., *Ber. chem. Ges.*, **31**, 1069 (1898).
7. Knorr, L., and Matthes, H., *Ber. chem. Ges.*, **34**, 3482 (1902).
8. Knorr, L., *Ber. chem. Ges.*, **22**, 2092 (1889).
9. Moyer, A. W., and du Vigneaud, V., *J. Biol. Chem.*, **143**, 373 (1942).
10. Stokes, J. L., Foster, J. W., and Woodward, C. R., *Arch. Biochem.*, **2**, 235 (1943).
11. Srb, A. M., and Horowitz, N. H., *J. Biol. Chem.*, **154**, 129 (1944).
12. Thompson, R. C., Isbell, E. R., and Mitchell, H. K., *J. Biol. Chem.*, **148**, 281 (1943).
13. du Vigneaud, V., *Harvey Lectures*, **38**, 39 (1942–43).
14. Jukes, T. H., and Oleson, J. J., *J. Biol. Chem.*, **157**, 419 (1945).

THE MECHANISM OF CARBON DIOXIDE FIXATION BY CELL-FREE EXTRACTS OF PIGEON LIVER: DISTRIBUTION OF LABELED CARBON DIOXIDE IN THE PRODUCTS*

BY HARLAND G. WOOD

(*From the Department of Physiology, University of Minnesota Medical School, Minneapolis*)

AND

BIRGIT VENNESLAND AND E. A. EVANS, JR.

(*From the Department of Biochemistry of the University of Chicago, Chicago*)

(Received for publication, March 22, 1945)

Cell-free pigeon liver extracts have been shown to catalyze a rapid fixation of $C^{11}O_2$ when incubated with pyruvate and fumarate as substrates (1). The net chemical change during the reaction consists of a conversion of fumarate to lactate and CO_2. The original pyruvate concentration remains unchanged, but very little $C^{11}O_2$ is fixed if pyruvate is omitted from the incubation mixture.

The available data indicated that fixation occurred as a result of the reversibility of the enzymic reactions:[1]

(1) $$CO_2 + CH_3{\cdot}CO{\cdot}COOH \rightleftharpoons COOH{\cdot}CH_2{\cdot}CO{\cdot}COOH$$

(2) $$COOH{\cdot}CH{:}CH{\cdot}COOH + H_2O \rightleftharpoons COOH{\cdot}CH_2{\cdot}CHOH{\cdot}COOH$$

(3) $$COOH{\cdot}CH_2{\cdot}CHOH{\cdot}COOH + CH_3{\cdot}CO{\cdot}COOH \rightleftharpoons COOH{\cdot}CH_2{\cdot}CO{\cdot}COOH + CH_3{\cdot}CHOH{\cdot}COOH$$

The reversibility of Reaction 2 can readily be demonstrated, and the reversibility of Reaction 3 is in complete accord with our knowledge of the behavior of such oxidation-reduction systems. Reaction 1 could only be demonstrated to occur in the direction of decarboxylation, as would be expected from the energy relations involved (3). Direct evidence of its reversibility, *i.e.*, of the formation of oxalacetate from pyruvate and CO_2 in pigeon liver, is lacking.

This paper presents evidence in favor of the proposed mechanism of fixation by proving that the fixed CO_2 is located in the carboxyl groups of

* That portion of the work reported in this paper carried out in the Department of Biochemistry, University of Chicago, was aided in part by grants from the John and Mary R. Markle Foundation and from the Dr. Wallace C. and Clara A. Abbott Memorial Fund.

[1] The possibility of a fourth, non-enzymic reaction, $C^{11}OOH{\cdot}CH{:}COH{\cdot}COOH \rightleftharpoons C^{11}OOH{\cdot}COH{:}CH{\cdot}COOH$, has been excluded by the results of Krampitz *et al.* (2), who have demonstrated that excess C^{13} in one carboxyl group of oxalacetate does not become spontaneously distributed in both carboxyl groups of the acid.

the acids present in the incubation mixture (lactic, pyruvic, malic, fumaric), as was previously surmised. On the other hand, attempts to demonstrate a reversal of the decarboxylation of oxalacetate by fixation of labeled carbon in the β-carboxyl group of oxalacetate indicated that such an exchange reaction is apparently too slow to function as the initial reaction in the rapid fixation of CO_2 which occurs in the presence of pyruvate and fumarate.

Methods

Dialyzed extracts of pigeon liver acetone powder were used in all experiments. The preparation of the enzyme, the methods of quantitative determination of the products, and the conditions used in conducting the reaction were the same as described by Evans, Vennesland, and Slotin (1), except that the method of Lehmann (4) was used for determination of lactate. $NaHC^{13}O_3$ was used instead of $NaHC^{11}O_3$ in the reaction mixture.

Separation and degradation of the products were accomplished by a modified form of the procedure described by Wood, Werkman, Hemingway, and Nier (5). The pyruvic acid was bound by bisulfite and the malic, fumaric, and lactic acids were separated from the pyruvic acid by extraction with ethyl ether. After removal of the bisulfite the pyruvic acid was recovered by ether extraction. The carboxyl group of pyruvate was then liberated by ceric sulfate oxidation (6).

The malic and fumaric acids were separated from the lactic acid by precipitation as the silver salts. The lactic acid was oxidized with permanganate to CO_2 and acetaldehyde. Malate and fumarate were separated from each other by virtue of the difference in their rates of extraction with ethyl ether and were then oxidized by permanganate.

A simpler procedure was used in Experiment II, Table I, no attempt being made to separate pyruvate from lactate, or malate from fumarate. The acid reaction products were extracted with ethyl ether and the malate and fumarate separated from the pyruvate and lactate by precipitation of the former as silver salts. Both fractions were then oxidized with permanganate in acid solution to CO_2 and acetaldehyde. The CO_2 originates from the carboxyl groups of all the acids present, while the acetaldehyde is derived from the α- and β-carbon atoms of lactic acid in the one fraction and from the α- and β-carbon atoms of malic acid in the second fraction. The exchange reactions with oxalacetate and $C^{13}O_2$ were carried out in a manner similar to that described by Krampitz, Wood, and Werkman (2) for the bacterial preparation. The dialyzed pigeon enzyme extract was activated with $MnCl_2$ and pyruvate was added to increase the rate of the carboxylation. When half the oxalacetate had disappeared the reaction mixture was deproteinized with one-third its volume of 10 per cent meta-

phosphoric acid. The precipitate was removed by centrifugation and the supernatant diluted with water to 30 ml. This is necessary in order to prevent the precipitation of aniline by the metaphosphoric acid. The $C^{13}O_2$ was removed by flushing for 15 minutes with a rapid current of CO_2 drawn through a sintered glass disk. The CO_2 left in solution was aerated into alkali with CO_2-free air, and analyzed for heavy carbon to ascertain the efficiency with which the removal of $C^{13}O_2$ had been accomplished. The oxalacetate was then decarboxylated with aniline citrate and the CO_2 representing the β-carboxyl group was collected in alkali.

Results

The results of the degradation studies prove conclusively that during incubation of pigeon liver extracts with pyruvate and fumarate all of the heavy carbon fixed is located in the carboxyl groups of the organic acids present in the reaction mixture. Table I summarizes the quantity of products found at the end of the reaction, the per cent excess C^{13} found in the various fractions, the per cent recovery of the total added C^{13}, and the per cent distribution of the total fixed C^{13}. In Experiment I the excess of C^{13} was the same, within experimental error, in the carboxyl groups of lactate and pyruvate. The isotope content of the carboxyl groups of malate and fumarate was likewise the same although the dicarboxylic acids contained more C^{13} than the monocarboxylic acids. In Experiment II the mono- and dicarboxylic acids contained the same per cent of excess C^{13}, 0.79 and 0.80 per cent. The fixation of CO_2 was quantitatively much greater in Experiment II than in Experiment I, 61.8 per cent of the added $C^{13}O_2$ being fixed in Experiment II as compared to 21.7 per cent in Experiment I. It seems probable that the reactions in Experiment II were more rapid than in Experiment I and the reversibility in this case was sufficient to give a complete distribution of the C^{13} in all carboxyl groups, while with the slower reaction rate an unequal distribution of C^{13} resulted. It was to be expected that the C^{13} concentration would be identical in the fumarate and malate, since the interconversion of these acids by fumarase occurs very rapidly. Likewise, since the lactate arises by reduction of pyruvate, the C^{13} concentration in the two compounds should be similar.

The attempts to demonstrate an exchange between $C^{13}O_2$ and the β-carboxyl group of oxalacetate did not yield conclusively positive results. A typical experiment is shown in Table II. The amount of C^{13} is practically within the limits of error of the method of measurement. These results should not be taken as an indication that exchange does not occur, but they do indicate that the rate of this exchange reaction is of a different order of magnitude than that of the rate of fixation of CO_2 with pyruvate and fumarate as substrates.

TABLE I

Distribution of Fixed $C^{13}O_2$ in Products of Reaction by Cell-Free Liver Enzyme

Experiment No.	Product		Excess C^{13}	Excess C^{13}	Recovery of added C^{13}	Distribution of fixed C^{13}
		mM	*per cent*	*mM*	*per cent*	*per cent*
I*	Pyruvate	3.80				
	·COOH		0.26	0.0099	7.1	32.6
	Lactate	1.57				
	·COOH		0.28	0.0044	3.1	14.5
	CH_3·CHOH·		0.02			
	Fumarate	0.52				
	·COOH		0.35	0.0036	2.6	11.8
	Malate	1.64				
	·COOH		0.38	0.0125	8.9	41.1
	·CHOH·CH_2·		0.00			
Totals				0.0304	21.7	
II†	Pyruvate and lactate	2.36				
	·COOH		0.79	0.0186	35.4	57.2
	CH_3·CHOH·		−0.01			
	Fumarate and malate	0.87				
	·COOH		0.80	0.0139	26.4	42.8
	·CHOH·CH_2·		0.00			
Totals				0.0325	61.8	

* 83 ml. of enzyme, 24 ml. of 0.005 M $MnCl_2$, 16 ml. of cozymase (1 mg. of crude preparation per ml.), 8 ml. of 0.5 M pyruvate, 8 ml. of 0.5 M fumarate, 8 ml. of 0.35 M $NaHC^{13}O_3$ containing 0.1403 mM excess C^{13}, 5 ml. of H_2O. Incubated 1[illegible] hours at 38° with no gas phase.

† 27 ml. of enzyme, 9 ml. of 0.005 M $MnCl_2$, 6 ml. of cozymase preparation, 3 ml. of 0.5 M pyruvate, 3 ml. of 0.5 M fumarate, 3 ml. of 0.35 M $NaHC^{13}O_3$ containing 0.0526 mM excess C^{13}, 6 ml. of H_2O. Incubated $1\frac{3}{4}$ hours at 39° with no gas phase.

TABLE II

$C^{13}O_2$ Exchange with Oxalacetate

	Washings	β-Carboxyl CO_2 from oxalacetate
CO_2, *mM*	0.74	0.30
Excess heavy carbon, %	0.01	0.03

5 ml. of dialyzed pigeon liver extract, 1.0 ml. of 0.01 $MnCl_2$, 1 ml. of 0.5 M pyruvate, 1 ml. of 0.5 M oxalacetate, 0.5 ml. of 0.35 M $NaHC^{13}O_3$ (heavy carbon 6.2 per cent). Incubated 25 minutes at 39°.

DISCUSSION

The results of the investigation of the distribution of the fixed carbon are in complete agreement with the proposed mechanism of fixation. However, these results give little information concerning the primary fixation reaction itself, other than to show that the CO_2 is fixed in a carboxyl group. The fact that the decarboxylation of oxalacetate in the pigeon liver preparations is enzymic makes plausible a reversal of this reaction, but the exchange studies indicate that such a reverse reaction is apparently too slow to account for the rapid fixation observed when fumarate is added to the pyruvate.

Failure to demonstrate an appreciable amount of exchange in the oxalacetate may, of course, be due to an inhibition of the reaction by the high concentrations of oxalacetate employed. During fixation of CO_2 in the presence of pyruvate and fumarate the concentration of oxalacetate is inappreciable. The possibility that the first product of fixation is not oxalacetate, but some other substance closely related to it, would also explain the observed low rate of exchange. Thus if the fixation reaction involved an intermediary X, in the following manner

$$\begin{array}{c} \text{Pyruvate} + CO_2 \rightleftarrows X \underset{-2H}{\overset{+2H}{\rightleftarrows}} \text{malate} \\ \qquad\qquad\quad \upharpoonleft\downharpoonright \\ \qquad\qquad\quad \text{Oxalacetate} \end{array}$$

and the conversion of X to oxalacetate occurred at a much slower rate than the reduction of X by lactate to form malate, one would expect little exchange between labeled CO_2 and oxalacetate to occur under our experimental conditions.

A comparison of the results obtained by Krampitz, Wood, and Werkman (2) with bacteria indicates that the pigeon liver and the bacterial preparations differ in several respects. Thus, the same procedure that resulted in a demonstration of fixation of CO_2 in oxalacetate by bacteria has not been successful with pigeon liver preparations.[2] The apparent direct formation of oxalacetate from pyruvate has also been demonstrated with the bacterial preparations by Kalnitsky and Werkman (7). However, a consideration of the energy relationships involved indicates that the amount of oxalacetate which could be formed from pyruvate and CO_2 by Reaction 1 would be too small to be detected by the methods employed (3). This fact alone would warrant the conclusion that the fixation mechanism involves at least one unknown step. In the case of the reaction in bacteria

[2] Krampitz, Wood, and Werkman obtained such results before the experiments described in this paper were undertaken.

the possibility of a phosphorylated intermediary has been suggested by Krampitz, Wood, and Werkman (2). No experimental evidence yet obtained indicates a participation of phosphate during CO_2 fixation in pigeon liver, however, although there is likewise no evidence that phosphate does not play a significant part in the reaction.

SUMMARY

The fixation of CO_2 by pigeon liver extracts, with pyruvate and fumarate as substrates, has been investigated with CO_2 labeled with C^{13}. The products of the reaction have been examined to determine the location and concentration of the fixed C^{13}. It has been demonstrated that the C^{13} is entirely in the carboxyl groups of the pyruvate, lactate, malate, and fumarate and that the concentration of C^{13} is approximately the same in all the carboxyl groups. The results are, therefore, in agreement with the previous proposals of Evans, Vennesland, and Slotin (1).

There remains some uncertainty concerning the details of the mechanism of the initial fixation reaction.

BIBLIOGRAPHY

1. Evans, E. A., Jr., Vennesland, B., and Slotin, L., *J. Biol. Chem.*, **147**, 771 (1943).
2. Krampitz, L. O., Wood, H. G., and Werkman, C. H., *J. Biol. Chem.*, **147**, 243 (1943).
3. Evans, E. A., Jr., *Harvey Lectures*, **39**, 273 (1943–44).
4. Lehmann, J., *Skand. Arch. Physiol.*, **80**, 237 (1938).
5. Wood, H. G., Werkman, C. H., Hemingway, A., and Nier, A. O., *J. Biol. Chem.*, **142**, 31 (1942).
6. Krebs, H. A., and Johnson, W. A., *Biochem. J.*, **31**, 645 (1937).
7. Kalnitsky, G., and Werkman, C. H., *Arch. Biochem.*, **4**, 25 (1944).

COMPOSITION OF BONE IN RELATION TO BLOOD AND DIET*

By ALBERT E. SOBEL, MORRIS ROCKENMACHER, and BENJAMIN KRAMER

(*From the Pediatric Research Laboratory and Division of Biochemistry, The Jewish Hospital of Brooklyn, New York*)

(Received for publication, December 27, 1944)

In the literature, various reports on bone analysis indicate that the inorganic composition of bone as represented by the formula $CaCO_3 \cdot n$-$[Ca_3(PO_4)_2]$ (in which as much as 6 per cent of the calcium present may be replaced by other bases (1)) often shows an excess of base over that required by this formula. This was shown by Gabriel in 1894 (2) and subsequently confirmed (3–5).

Though many attempts have been made to demonstrate the presence of anions in excess of the above formula (5), this fact has not yet been established. Such evidence might support a theory proposed by Shear and Kramer (6–8) and confirmed by Logan (9) that $CaHPO_4$ is the first solid aggregate formed in calcification.

To investigate this problem further, our previous experiments on the relationship of diet and blood to bone carbonate (10) were extended to include total base and phosphate studies, since in those experiments bloods containing high phosphorus and low calcium levels and low phosphorus and high calcium levels were obtained. In six out of seven experimental groups of rats, an excess of base over that required by the empirical formula for bone composition was observed, whereas in one group the opposite feature was noted.

EXPERIMENTAL

The experimental procedure and the diets employed are fully described in the previous paper (10). The femora from the final experiments described in that paper were employed. In addition to calcium and carbonate, these bones were further analyzed for phosphorus, total base, and ammonia. The methods employed for the analyses are those described by the authors (11).

* Preliminary paper presented before the American Society of Biological Chemists (*Federation Proc.*, **2**, 70 (1943)). Partially presented before the Division of Biological Chemistry, 107th Meeting of the American Chemical Society, Cleveland, Ohio, April 3–7 (1944).

Calculations

mM Ca = (mg. total Ca in bone)/40.08
" P = (" " P " ")/31.02
" CO_2 = (" " CO_2 " ")/60.01

Milliequivalents total base = milliequivalents total base in bone minus milliequivalents NH_4^+ in bone

Residual Ca = mM Ca in bone minus mM CO_2 in bone
" total base = milliequivalents total base in bone − (2 × mM CO_2)

% Ca, % P, % CO_2 = mg. Ca, P, or CO_2 × 100/mg. weight of fat-free dried femur

Milliequivalents total base per 100 mg. = milliequivalents total base × 100/mg. weight of fat-free dried femur

The ash may be calculated by adding

Mg. Ca + [mg. P × PO_4:P] + mg. CO_2 +24 [milliequivalents total base − (2 × mM Ca)]

PO_4:P = (31.02 + 64.0)/31.02 = 3.063

The excess of total base over calcium is mainly Mg + Na.

n was calculated by three methods:

$n_1 = \frac{1}{3}$ residual Ca/mM CO_3
$n_2 = \frac{1}{6}$ " total base/mM CO_3
$n_3 = \frac{1}{2}$ mM P/mM CO_3

TABLE I

Composition of Serum (Mean Values)

Group	Ca	P	CO_2	Ca x P	Ca:P	P:Ca	P:CO_2
	mg. per cent	*mg. per cent*	*vol. per cent*				
Reference	11.2	10.3	51.6	115	1.12	0.92	0.200
A	6.3	8.9	58.8	56	0.71	1.41	0.151
B	11.8	4.2	59.0	50	2.81	0.36	0.071
C	6.0	9.4	56.9	56	0.64	1.57	0.165
A + vitamin D	8.7	8.9	54.7	77	0.98	1.02	0.163
B + " "	13.3	6.8	57.1	90	1.96	0.51	0.119
C + " "	8.8	9.3	52.6	82	0.95	1.06	0.177

The mean results of the bone analyses were evaluated by the statistical methods of Fisher as applied to small samples (12). *P* represents the frequency with which the difference between two means may be due to chance alone. When *P* is 0.05 or less, the difference between the means is considered statistically significant.

Composition of Blood Serum—The results obtained in serum analysis for Ca, inorganic P, and CO_2 are shown in Table I. The significance of these results has been discussed previously (10). They are reproduced here because frequent references will be made to them.

Deposition of Inorganic Components As Indicated by Total Amount Present in Femur—Table II shows the absolute amount of calcium, phos-

TABLE II

Absolute Composition of Bone (Mean Values)

Group	Weight of bone	Ca	P	CO_2	Total base	Residual Ca	Residual total base
	mg.	*mM × 1000*	*mM × 1000*	*mM × 1000*	*m.eq. × 1000*	*mM × 1000*	*m.eq. × 1000*
Reference	40.59	153.72	92.36	13.89	312.51	139.83	284.73
A	74.45	189.30	114.16	20.96	387.43	168.34	345.51
B	93.41	297.90	168.30	45.25	606.21	252.65	515.71
C	69.44	196.44	131.91	23.28	406.70	173.16	360.25
A + vitamin D	81.63	235.40	141.58	23.49	482.99	211.91	436.87
B + " "	129.77	546.30	308.70	75.77	1127.24	472.20	975.70
C + " "	77.38	242.30	146.60	23.91	491.46	218.39	443.64
P, A *vs.* B		$<10^{-7}$	$<10^{-7}$	$<10^{-10}$	$<10^{-8}$		
" " " C		0.71	0.12	0.15	0.54		
" B " "		$<10^{-5}$	0.02	$<10^{-10}$	$<10^{-6}$		
" A " A + vitamin D		0.043	0.04	0.32	0.04		
" B " B + " "		0.015	$<10^{-8}$	$<10^{-6}$	$<10^{-7}$		
" C " C + " "		0.051	0.26	0.76	0.04		
" A + vitamin D *vs.* B + vitamin D		$<10^{-4}$	$<10^{-10}$	$<10^{-10}$	$<10^{-10}$		
P, A + vitamin D *vs.* C + vitamin D		0.78	0.68	0.88	0.86		
P, B + vitamin D *vs.* C + vitamin D		$<10^{-4}$	$<10^{-10}$	$<10^{-10}$	$<10^{-10}$		
P, reference *vs.* A		0.08	0.074	$<10^{-5}$	0.066		
" " " B		Sig.	Sig.	Sig.	Sig.		
" " " C		0.075	<0.01	$<10^{-9}$	0.029		
" reference *vs.* A + vitamin D		$<10^{-3}$	Sig.	Sig.	Sig.		
P, reference *vs.* B + vitamin D		Sig.	"	"	"		
P, reference *vs.* C + vitamin D		"	"	"	"		

P indicates the probability that the difference between the two means is due to chance.

Sig. indicates that the difference between the two means is statistically significant. This method was employed in comparing the Reference group against the others. These values were not calculated because, by inspection of the differences between the means and $S(x-\bar{x})^2$, *P* would be lower than the next lowest *P* (see Fisher (12)). x = individual observation; $\bar{x}$ = mean value; $x - \bar{x}$ = deviation from the mean; $S(x - \bar{x})$ = sum of the deviations from the mean.

phorus, carbonate, total base, and ammonia deposited. The total weight of the bone and the calculated residual calcium and residual total base are also shown.

It is readily seen that the total base in all cases is higher than the calcium

expressed as milliequivalents (calcium in milliequivalents = 2 times the calcium in mM). This is in agreement with the findings of Logan (1) and Kramer *et al.* (13) and others. All the calcium, phosphorus, and carbonate values were higher than those of the reference group, which gives the initial content of these components in the bones of the group. The greatest increases in the deposition were on the high calcium-low phosphorus diets.

The addition of calcium to Diet A increased the amount of phosphorus deposited compared to that found in Group A to a statistically significant degree, whereas the addition of phosphorus to Diet A (Diet C) was without effect.

Vitamin D caused a statistically significant increase in all bone components analyzed in Group B. In Group A, there were increases in two out of the three main bone components; namely, total base and phosphorus (since calcium is the principal component of the total base of the bone, it need not be considered independently). In Group C, there was a statistically significant increment only in the total base.

Deposition of Inorganic Components As Indicated by Percentage Present in Femur—It may be seen in Tables II and III that, whereas the absolute amounts of total base and phosphorus were higher in all groups than in the reference group, the percentage values were lower in all groups except in Group B receiving vitamin D.

In the absence of vitamin D, Group B has a distinctly higher percentage of carbonate than the other two groups, but the difference in the percentages of base and phosphorus is without significance. There were no statistically significant differences between Groups A and C with regard to the percentage values of the inorganic components studied.

In the presence of vitamin D, Group B had higher percentages of the inorganic components than the other two groups. There were again no statistically significant differences between Groups A and C.

Vitamin D increased the density of the bones as measured by the percentage of ash in Groups B and A but not in Group C.

Composition of Bone Calculated from Data—The residual total base to P ratios are given in Table IV. The theoretical ratio is 1.50 from the formula $n[Ca_3(PO_4)_2]\cdot CaCO_3$. It is seen in Table IV that the total base was in excess of this in six out of seven groups. In the high phosphorus group, in the absence of vitamin D this ratio was 1.36. This variation from the usually reported ratio of 1.50 is unmistakably significant statistically. The formula $m(CaHPO_4)\cdot n[Ca_3(PO_4)_2]\cdot CaCO_3$ is necessary to explain this unusual ratio, where $m = 2$ and $n = 1.6$.

n was calculated by three methods and n_1 ranged from 1.88 to 3.34, n_2 from 1.92 to 3.41, and n_3 from 1.86 to 3.33. If we examine the relationship between the blood composition and n (see Tables I and IV), there is a

distinct correlation between the serum P:CO_2 ratio and n_3, except for the third and fourth values in the series, which become respectively the fourth and third. The correlation is not as good for n_1 and n_2 because Group C falls out of line; this may be explained on the basis of the unusual residual total base to P ratio found in the bones of this group.

TABLE III

Composition of Bone (Mean Values)

Group	Ash	Ca	P	CO_2	Total base	NH_3
	per cent	*per cent*	*per cent*	*per cent*	*m.eq. per 100 mg.*	*m.eq. per 100 mg.*
Reference	39.15	15.26	7.11	2.08	777.0	5.57
A	27.01	10.39	4.99	1.74	531.1	4.20
B	33.39	13.02	5.66	2.82	657.0	3.05
C	32.18	11.45	5.97	2.04	590.2	4.60
A + vitamin D	30.51	11.80	5.47	1.80	599.8	3.80
B + " "	43.61	17.00	7.48	3.53	877.5	2.55
C + " "	31.77	12.50	5.86	1.85	634.6	2.82
P, A *vs.* B		0.047	0.20	$<10^{-4}$	0.036	0.064
" " " C		0.59	0.12	0.09	0.20	0.58
" B " "		0.24	0.60	0.018	0.25	0.07
" A " A + vitamin D		0.38	0.43	0.73	0.11	0.48
" B " B + " "		$<10^{-5}$	$<10^{-10}$	0.017	$<10^{-5}$	0.45
" C " C + " "		0.35	0.82	0.28	0.32	0.04
" A + vitamin D *vs.* B + vitamin D		$<10^{-9}$	$<10^{-5}$	$<10^{-10}$	$<10^{-9}$	0.06
P A + vitamin D *vs.* C + vitamin D		0.50	0.46	0.78	0.49	0.15
P B + vitamin D *vs.* C + vitamin D		$<10^{-9}$	$<10^{-7}$	$<10^{-10}$	$<10^{-10}$	0.7
P reference *vs.* A		Sig.	$<10^{-5}$	0.66	Sig.	0.60
" " " B		0.04	10^{-3}	0.02	0.03	0.41
" " " C		Sig.	0.04	0.76	Sig.	0.29
" " " A + vitamin D		"	$<10^{-3}$	0.07	"	0.15
" " " B + " "		$<10^{-2}$	0.17	$<10^{-10}$	$<10^{-2}$	0.12
" " " C + " "		$<10^{-4}$	$<10^{-4}$	0.10	$<10^{-4}$	0.09

See note below Table II.

Table V shows the increments in the components and composition of the bone during the experimental period. For Table V, the values found in the reference group were subtracted from those found in the experimental groups at the end of the experimental period. This method of evaluating the data may be criticized on the ground that the composition of the bone during the experimental period was not static, but that there was a continual turnover of old bone salts and replacement by new bone salts, as

has been clearly indicated by experiments on bone deposition with radioactive phosphorus (14–16). This approach is especially apt to mislead one when the net increase in deposition is relatively small in magnitude.

TABLE IV

Composition of Bone (Mean Values)

The results represent molar ratios.

Group	$\frac{CO_2}{\text{Total Ca}}$	$\frac{CO_2 \times 2}{\text{Total base}}$	$\frac{\text{Residual Ca}}{P}$	$\frac{\frac{1}{2}\text{ residual total base}}{P}$	$n_1, \frac{\frac{1}{2}\text{ residual Ca}}{CO_2}$	$n_2, \frac{\frac{1}{2}\text{ residual total base}}{CO_2}$	$n_3, \frac{\frac{1}{2}P}{CO_2}$
Reference	0.0910	0.0894	1.51	1.54	3.34	3.41	3.33
A	0.1112	0.1084	1.48	1.52	2.68	2.76	2.72
B	0.1526	0.150	1.51	1.54	1.88	1.92	1.86
C	0.1175	0.1149	1.33	1.36	2.52	2.58	2.82
A + vitamin D	0.1018	0.0993	1.49	1.55	2.98	3.08	3.01
B + " "	0.1389	0.1351	1.52	1.57	2.07	2.15	2.04
C + " "	0.0981	0.09658	1.49	1.51	3.11	3.15	3.06
A *vs.* B			0.263		$<10^{-9}$		
B " C			$<10^{-9}$		$<10^{-7}$		
A " "			$<10^{-7}$		0.17		
A " A + vitamin D			0.63		0.04		
B " B + " "			0.63		0.046		
C " C + " "			$<10^{-9}$		$<10^{-4}$		
A + vitamin D *vs.* B + vitamin D			0.049		$<10^{-9}$		
A + vitamin D *vs.* C + vitamin D			1.00		0.49		
B + vitamin D *vs.* C + vitamin D			0.19		$<10^{-9}$		
Reference *vs.* C			$<10^{-9}$				
" *vs.* C + vitamin D					0.11		
Reference *vs.* A + vitamin D					0.01		

The residual total base to P ratio in the absence of vitamin D indicates an excess of acid over base deposited in Groups A and B. In Group C, this ratio is 0.955. The ratio for $CaHPO_4$ is 1.00. Thus, the empirical composition of the salt being deposited as represented by the increment may be considered as such a compound. By this reasoning, some $CaHPO_4$ must also be present in the bones of rats in Group A. In contrast, Group B has a ratio of 1.58, indicating that there is a possibility of a $Ca(OH)_2$ type

of compound being deposited during the experimental period. In the presence of vitamin D, the increment in the residual total base to P ratio of the salt being deposited was highest in Group B and lowest in Group C, Group A being intermediate. There is an excess of base in the bones of Groups A and B. The influence of vitamin D was manifest in both high phosphorus-low calcium groups (Groups A and C) by a marked increase in bone residual total base to P ratio. In Group B, in which this ratio was high, the influence of vitamin D was negligible.

The relationship between blood composition and increment in the residual total base to P ratios (see Tables I and V) shows a distinct correlation between the serum Ca:P ratios and the increment in residual total base

TABLE V

Increments in Inorganic Composition of Bone during Experimental Period

Group	Increment in weight of bone	Ca	P	CO_3	$\frac{\text{Residual total base}}{\text{P}}$	$\frac{\text{Residual Ca}}{\text{P}}$	n_1, $\frac{\frac{1}{2}\text{ residual Ca}}{CO_3}$	n_3, $\frac{P}{2 \times CO_3}$
	mg.	*mM* × *1000*	*mM* × *1000*	*mM* × *1000*		*mM* × *1000*	*mM* × *1000*	*mM* × *1000*
A	23.7	35.58	21.80	7.07	1.39	1.31	1.34	1.54
B	45.0	144.18	75.94	31.36	1.58	1.49	1.20	1.21
C	17.2	42.72	39.55	9.39	0.955	0.842	1.19	2.11
A + vitamin D	46.4	81.68	49.22	9.60	1.55	1.47	2.50	2.56
B + " "	49.2	392.58	216.34	61.88	1.59	1.54	1.79	1.75
C + " "	40.5	88.58	54.24	10.02	1.46	1.45	2.61	2 69

to P, except for the first and second values in the series, which become respectively second and first. However, the difference between the increment in the residual total base to P ratio of the first and second largest value is negligible (first = 1.59, second = 1.58).

The values in the increment of $n_3(\frac{1}{2}P):CO_3$ are related to serum $P:CO_2$ ratios. If the serum $P:CO_2$ ratios are arranged in a series for all the groups, the increment in n_3 follows the same order as the serum $P:CO_2$ ratios (see Tables I and V), except for the second and third values in the series in which the serum $CO_2:P$ ratio is 0.163 and 0.165 and n_3 is 2.56 and 2.11 respectively. However, the difference between 0.163 and 0.165 is negligible. Group B has the lowest increment in n_3, both in the presence and absence of vitamin D. Group C showed the highest n_3 values with and without vitamin D.

If we consider the increment of n_1 in the same fashion, Group C falls out

of line. This, however, can be explained by the great excess of phosphate that was found in the bone, or, conversely, by the deficiency of base. Thus, this fact provides an explanation of the lack of conformity of Group C when evaluated by the CO_3:Ca ratio only, as noted in our previous paper (10).

The influence of vitamin D on the increment of both n_1 and n_3 was marked. There was an increase in all cases. The extent of this increase is greater than that expected from the increase in serum P:CO_2 ratios. This may be related to an increase of Ca $\times$ P product in the serum (see Tables I and V), although some other factor, playing an auxiliary rôle, cannot be excluded. An increase in serum Ca $\times$ P product would favor increased calcium phosphate deposition (as compared with the amount of $CaCO_3$ deposited). This would result in lower values of n_1 and n_3.

DISCUSSION

Composition of Bone and Mechanism of Its Formation—In the literature, there is general agreement that the composition of bone salts undergoes wide variations (9, 17, 18). In spite of these observed variations, however, all the x-ray evidence to date indicates that the bone salts are present in an apatite structure (18). Apatite may be considered as a continuous series of solid solutions in which the composition of the solid reflects the composition of the liquid with which it is in equilibrium. There is an added factor that must be considered in accounting for the variations in bone salts; namely, adsorption. The small size of the bone crystals offers large surfaces which provide ideal conditions for adsorption. The experiments of Logan and Taylor (19) indicate that the calcium carbonate portion of the bone may be adsorbed or at least present in higher concentration at the surface than in the interior of the bone. The *in vitro* experiments of Hodge *et al.* (20) with radioactive sodium indicate the operation of an adsorption isotherm in the uptake of sodium by bone and also indicate that large amounts of phosphate and fluoride may be adsorbed. However, the possibility of isomorphous replacement of sodium in the apatite lattice has not been eliminated.

It is not yet possible to decide whether deposition of bone salt occurs as a result of the formation of solid solutions or is a two stage process consisting of the formation of an initial precipitate followed by ion exchange and adsorption. In both of the above processes, the composition of the solid changes with changes in the composition of the liquid phase. Similarly, variations in the composition of bone salt may be correlated with changes in the composition of the serum. There was an almost direct relationship between the bone P:CO_3 (n_3) ratio and the serum P:CO_2 ratio. These relationships become even more evident if the blood composition is

considered throughout the experiment. The more extreme the dietary Ca:P, the more rapidly the changes in serum composition take place. This was shown in the experiments of Boelter and Greenberg (21) for low calcium-high phosphorus diets and in unpublished experiments in this laboratory for high calcium-low phosphorus diets. Vitamin D tends to prevent these changes by preventing the fall of either serum calcium or inorganic phosphorus. Vitamin D may also have an effect on the operation of the "local factors" in the bone. Consequently, the comparison of experiments in which vitamin D is the only differing feature between two groups is open to this limitation. In general, the increased $P:CO_3$ ratio of the bones of the vitamin D-fed group was higher than that expected from the changes in the serum $P:CO_2$ ratios alone. These increases, however, were accompanied by higher values of the serum calcium and consequently the $Ca \times P$ products were also higher.

The correlation between the composition of serum and of bone was even better when only the net *increments in composition* of the bone (during the experimental period) are considered. (The limitations of this method of reasoning were pointed out in the experimental portion of the paper.) There was an almost direct relationship between serum $P:CO_2$ and increment in bone $P:CO_3$ (n_3) when each series was arranged in the order of its magnitude. In addition, there was a direct relationship (with one doubtful exception) between serum Ca:P and bone residual Ca:P (or residual total base to P) when each series was arranged in the order of its magnitude. The above relationships are similar to that indicated by Logan and Taylor (see Table IV (22)) between the composition of calcium phosphate precipitates and that of the supernatant fluids.

An exact quantitative relationship between the composition of blood and bone cannot be expressed, since this would involve a knowledge of the activities of the bone-forming ions at the site of deposition. Such knowledge is not yet available. These experiments, however, do indicate that the composition of the serum is one of the controlling factors. Another factor is one which affects diffusion to the site of deposition which may be influenced by vitamin D. The various "local factors" may also be instrumental in changing the composition of the fluid. For example, vitamin D has been shown to influence the conversion of organic to inorganic phosphate in the tissues (15). If this holds true for the bone cell also, then the increased $PO_4:CO_3$ ratio of bone would be over and above that represented by serum values. The presence of enzymatic factors which have been shown to play a part in calcification (23–25) must also be considered. Experiments on calcification *in vitro* have shown that a minimal $Ca \times P$ product is required for deposition (26). This minimal $Ca \times P$ product was lowest in embryonic rabbit bone (27). In rachitic animals, a higher product

was necessary for new calcification, and the more prolonged the rachitic period, the higher the product required (28). In rickets due to beryllium and strontium the required product is much higher (29, 30). The experiments with strontium rickets indicated a reversible injury to a "local factor," enzymatic in nature, other than phosphatase. The more recent experiments of Gutman *et al.* (25) indicate that the enzyme system for "phosphorylative glycolysis" is important in the calcification of bone cartilage.

From this discussion, it is evident that the "local factors" must be considered in lime salt deposition. "Local factors" in themselves operate within the limits prescribed by the physicochemical factors. In each case, a minimal Ca $\times$ P product is required and the composition of the bone is a reflection of the composition of the serum. This is dramatically brought out in the bones of Group C, which had the empirical composition of $[Ca_3(PO_4)_2]_{2.0}(CaHPO_4)_{1.6} \cdot CaCO_3$. Precipitates of such composition were obtained by Logan and Taylor (22) from high phosphate-low calcium solutions. The usual blood composition does not have a sufficiently high phosphorus concentration with respect to the calcium concentration for the formation of such precipitates. With the extremely high phosphorus-low calcium diet (Diet C), however, high inorganic phosphorus and low calcium levels in the serum are rapidly obtained and maintained. There was no decrease in the carbonate content of these bones because the absolute amount of carbonate was actually higher than at the beginning of the experimental period. An examination of the data of Boelter and Greenberg (21) shows bones with similar composition following calcium-deficient diets.

The importance of $CaHPO_4$ in bone has been emphasized in discussions on the physicochemical mechanism of bone salt deposition. Wendt and Clarke (31) were the first to suggest that $CaHPO_4$ is a step in the formation of tertiary calcium phosphate. Shear and Kramer (6) then suggested that this was the first step in the formation of bone salts. They found serum to be just saturated or slightly undersaturated with respect to $CaHPO_4$ (7, 8). Calcification is not obtained either *in vivo* or *in vitro* when the ion product $[Ca^{++}] \times [HPO_4^{=}]$ is markedly below the solubility product of $CaHPO_4$ (7). Logan and Taylor (22), in a study of the course of precipitation of calcium phosphates, found that after the initial mixing there was some $CaHPO_4$ present in the precipitate. On prolonged equilibration, the composition of the precipitate underwent a change. In solutions containing excess calcium, the $CaHPO_4$ disappeared, apparently being replaced by $Ca_3(PO_4)_2$ and $Ca(OH)_2$. In solutions containing an excess of phosphate, some of the $CaHPO_4$ remained after prolonged equilibration. They concluded that $CaHPO_4$ is the first aggregate formed in the deposition of bone salt. This first solid removed calcium when in contact with solutions

containing excess calcium to form $Ca_3(PO_4)_2$. In the presence of a solution containing excess phosphate, phosphate is lost to the solution from the precipitate with the formation of $Ca_3(PO_4)_2$. Calcium, carbonate, and hydroxyl ion may be removed from solution even when their concentrations are such that the ion products $[Ca^{++}][CO_3^{-}]$ and $[Ca^{++}][OH^{-}]^2$ are less than the solubility products of $CaCO_3$ and $Ca(OH)_2$. "The composition of the precipitates after long equilibration depends on the composition of the liquid phase" (22).

The main arguments against the $CaHPO_4$ theory are summarized by Huggins (17). In the early calcification in healing rickets, Kramer and Shear (5) found not a low bone residual Ca:P ratio but a high value, indicating the presence of $Ca(OH)_2$. This argument may be answered by pointing out that the serum P:CO_2 ratio is low in rickets due to a high calcium-low phosphorus diet, thus accounting for the deposition noted. It is seen in our experiments that on such diets, even in the presence of vitamin D, there is a relatively low serum phosphorus and an excess of base in the bone over that required by the formula $Ca_3(PO_4)_2$. Had similar observations been made on high phosphorus-low calcium rickets, entirely different results might have been obtained. Another argument advanced against the acid phosphate theory is that $CaHPO_4$ has never been demonstrated by x-ray methods of analysis. Small amounts of this compound would not be revealed by the techniques hitherto employed. If $CaHPO_4$ was present at the surface of the crystal, it would not be revealed, according to Walden and Cohen (32). If the $CaHPO_4$ were part of the apatite lattice (of the general structure $Ca_3(PO_4)_2 \cdot CaX$, in which part of X is HPO_4^{-}), x-rays would again not reveal its presence in bone. Chemical analysis, however, would empirically show the existence of $CaHPO_4$ as such in bone. Hitherto, such chemical proof has not been presented, even though evidence was already present in the bones of the experimental rats of Boelter and Greenberg (21), who did not recognize this fact and whose analyses were limited to calcium and phosphorus only. In our experiments, bones with the average inorganic composition of $[Ca_3(PO_4)_2]_{2.0}$-$(CaHPO_4)_{1.6} \cdot CaCO_3$ were obtained. Whether $CaHPO_4$ is present in our bone salts as an independent crystal we are not prepared to state, but we hope to undertake such a study, using x-ray analysis. The third criticism of the acid phosphate theory was raised by Morgulis and Janecek (4); namely, that any $CaHPO_4$ present would be converted to pyrophosphate on incineration and would have to be reconverted by acid hydrolysis. No such pyrophosphate was found by the above authors. It would be interesting to see whether the bones of Group C give pyrophosphates under such conditions.

Recently, Greenwald, Redish, and Kibrick (33) presented evidence for the existence of undissociated $CaHPO_4$ in solution. They calculate that

the value of the dissociation constant of $CaHPO_4$ indicates that in plasma at pH 7.35 and $\mu = 0.152$, containing 1 mM of phosphate and 1.25 mM of calcium not combined with protein, approximately 0.055 mM of $CaHPO_4$ is present in solution. In subsequent work, Greenwald (34) concurs with Shear and Kramer (6–8) and Logan and Taylor (22) that, if the solubility product of $CaHPO_4$ is exceeded, the immediate precipitate appears to be $CaHPO_4$. He found that it is only later that the composition of the precipitate may change to approximate that of $Ca_3(PO_4)_2$.

The work of Shear and Kramer (6–8), Logan and Taylor (22), and Greenwald (34) has been cited to show the increasing consideration which is being given to the rôle of $CaHPO_4$ in calcification. Our data do not enable us to choose between these proposed mechanisms of calcification. Our work, however, serves to eliminate one of the major criticisms of one of these theories of calcification and supports this approach by indicating the probable existence of $CaHPO_4$ in bone under certain conditions. By simultaneous study of blood and bone composition, it has been possible to show that the final composition of bone does depend on that of the liquid phase in contact with it. The case for regarding the phenomenon of calcification as a physicochemical process has been strengthened and another step towards clarification of the mechanism involved has been made.

Once again, in another manner, has the importance of dietary calcium and phosphorus been shown. The composition of bone is related to serum $P:CO_2$ ratios, Ca:P ratios, and Ca × P products. These ratios and products, in turn, are influenced by the dietary calcium to phosphorus ratios and absolute levels, modified by dietary vitamin D (35–37).

SUMMARY

1. The composition of bone as represented by the formula $CaCO_3 \cdot n[Ca_3(PO_4)_2]$ (in which some of the calcium present may be replaced by other bases) showed an excess of base over that required by this formula in six out of seven of the experimental groups of rats examined. The bone residual total base to P ratio ranged from 1.52 to 1.57 and the bone residual Ca:P ratio ranged from 1.48 to 1.52 (theoretical bone residual total base to P ratio = 1.50).

2. The presence of excess phosphate over that required by the above formula for bone composition was demonstrated in one of our experimental groups, indicating an empirical composition of $[(CaHPO_4)_{1.6}(Ca_3(PO_4)_2)_2 \cdot CaCO_3]$. The bone residual total base to P ratio was 1.36 and the residual Ca to P ratio was 1.33.

3. n as measured by the $PO_4:CO_3$ ratio in the bone appears to be correlated to the serum inorganic $P:CO_2$ ratios. n ranged from 1.86 to 3.33 in the various experimental groups.

4. It has been shown again that the composition of the diet is reflected in the blood serum and that the composition of the bone is related to that of the serum.

5. The significance of these findings in the mechanism of the deposition of bone salt has been discussed.

BIBLIOGRAPHY

1. Logan, M. A., *J. Biol. Chem.*, **110**, 375 (1935).
2. Gabriel, S., *Z. physiol. Chem.*, **18**, 257 (1894).
3. Klement, R., *Z. physiol. Chem.*, **184**, 132 (1928).
4. Morgulis, S., and Janecek, E., *J. Biol. Chem.*, **93**, 455 (1931).
5. Kramer, B., and Shear, M. J., *J. Biol. Chem.*, **79**, 147 (1928).
6. Shear, M. J., and Kramer, B., *J. Biol. Chem.*, **79**, 125 (1928).
7. Shear, M. J., Washburn, M., and Kramer, B., *J. Biol. Chem.*, **83**, 697 (1929).
8. Shear, M. J., and Kramer, B., *J. Biol. Chem.*, **86**, 677 (1930).
9. Logan, M., *Physiol. Rev.*, **20**, 522 (1940).
10. Sobel, A. E., Rockenmacher, M., and Kramer, B., *J. Biol. Chem.*, **158**, 475 (1945).
11. Sobel, A. E., Rockenmacher, M., and Kramer, B., *J. Biol. Chem.*, **152**, 255 (1944).
12. Fisher, R. A., Statistical methods for research workers, Edinburgh, 6th edition, 128 (1936).
13. Kramer, B., Yuska, H., and Steiner, M. M., *Am. J. Dis. Child.*, **57**, 1044 (1939).
14. Chievitz, O., and Hevesy, G., *K. Danske Vidensk. Selsk., Biol. Medd.*, **13**, No. 9 (1937).
15. Cohn, W. E., and Greenberg, D. M., *J. Biol. Chem.*, **130**, 625 (1939).
16. Shimotori, N., and Morgan, A. F., *J. Biol. Chem.*, **147**, 201 (1943).
17. Huggins, C., *Physiol. Rev.*, **17**, 119 (1937).
18. Eisenberger, S., Lehrman, A., and Turner, W. D., *Chem. Rev.*, **26**, 257 (1940).
19. Logan, M. A., and Taylor, H. L., *J. Biol. Chem.*, **125**, 391 (1938).
20. Hodge, H. C., Koss, W. F., Ginn, J. T., Falkenheim, M., Gavett, E., Fowler, R. C., Thomas, I., Bonner, J. F., and Dessauer, G., *J. Biol. Chem.*, **148**, 321 (1943).
21. Boelter, M. D. D., and Greenberg, D. M., *J. Nutr.*, **21**, 61 (1941).
22. Logan, M., and Taylor, H. L., *J. Biol. Chem.*, **125**, 377 (1938).
23. Robison, R., The significance of phosphoric esters in metabolism, New York (1932).
24. Kay, H. D., *Physiol. Rev.*, **12**, 384 (1932).
25. Gutman, A. B., Warrick, F. B., and Gutman, E. B., *Science*, **95**, 461 (1942).
26. Shipley, P. G., Kramer, B., and Howland, J., *Biochem. J.*, **20**, 379 (1926).
27. Niven, J. S. F., and Tobison, R., *Biochem. J.*, **28**, 708 (1934).
28. Rosenheim, A. H., *Biochem. J.*, **28**, 708 (1934).
29. Sobel, A. E., Cohen, J., and Kramer, B., *Biochem. J.*, **29**, 2640, 2646 (1935).
30. Sobel, A. E., Goldfarb, A. R., and Kramer, B., *J. Biol. Chem.*, **108**, 395 (1935).
31. Wendt, G. L., and Clarke, A. H., *J. Am. Chem. Soc.*, **45**, 882 (1923).
32. Walden, G. H., Jr., and Cohen, M. U., *J. Am. Chem. Soc.*, **57**, 2591 (1935).
33. Greenwald, I., Redish, J., and Kibrick, A. C., *J. Biol. Chem.*, **135**, 65 (1940).
34. Greenwald, I., *J. Biol. Chem.*, **143**, 711 (1942).
35. Bethke, R. M., Kick, C. H., and Wilder, W., *J. Biol. Chem.*, **98**, 389 (1932).
36. Brown, H. B., Shohl, A. T., Chapman, E. E., Rose, C. S., and Saurwein, E. M., *J. Biol. Chem.*, **98**, 207 (1932).
37. Kramer, B., and Howland, J., *J. Nutr.*, **5**, 39 (1932).

FURTHER STUDIES ON FACTORS THAT AFFECT XANTHURENIC ACID EXCRETION*

BY E. C. MILLER AND C. A. BAUMANN

(From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison)

(Received for publication, April 2, 1945)

Previous reports have shown that rats (1) and mice (2) deficient in pyridoxine excrete xanthurenic acid in the urine when fed diets containing tryptophane. In this paper data are presented on the excretion of administered xanthurenic acid by normal and pyridoxine-deficient animals. Studies are also included on the relative effectiveness of pyridoxal and pyridoxamine in promoting growth and in preventing the excretion of xanthurenic acid.

Methods

Rats and mice were kept in screen bottom cages in groups of two and four respectively. Food and water were given *ad libitum*, and the animals were weighed at weekly intervals. Fresh diets were mixed for each series and stored at 5°. The diets for the mice contained 10 or 45 per cent of purified casein,[1] 2 per cent of corn oil (Mazola), 4 per cent of Wesson's salt mixture (3), and glucose[2] to 100 per cent. The corn oil contained 0.1 per cent of halibut liver oil, and the following vitamins were added to each gm. of diet: 3.3 γ of thiamine hydrochloride, 10.0 γ of nicotinic acid, 13.3 γ of calcium pantothenate, 6.6 γ of riboflavin, 333 γ of inositol, 200 γ of *p*-aminobenzoic acid, 166 γ of choline chloride, and 0 to 10 γ of pyridoxine hydrochloride.[3] For the experiments with rats the diets contained 10 or 45 per cent of purified casein, 5 per cent of corn oil, 4 per cent of Wesson's salt mixture, and glucose to 100 per cent. To each gm. of diet were added 3 γ of nicotinic acid, 3 γ of thiamine hydrochloride, 13.2 γ of calcium pantothenate, 4.5 γ of riboflavin, 1 mg. of choline chloride, and 0 to 10 γ of pyridoxine hydrochloride. Each rat received 1 drop of halibut liver oil per week. In certain experiments pyridoxal, pyridoxamine, or the lactone of 2-methyl-3-

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by the Wisconsin Alumni Research Foundation and the Jonathan Bowman Fund for Cancer Research.

[1] Crude casein extracted for 5 days with several changes of tap water, and then extracted with two changes of ethyl alcohol for 4 days at 50°.

[2] Cerelose, a pure commercial glucose monohydrate.

[3] The terms pyridoxine and pyridoxine hydrochloride are used interchangeably, and all values are expressed as the hydrochloride.

hydroxy-4-hydroxymethyl-5-carboxypyridine was substituted for pyridoxine hydrochloride.[4] In other experiments rats or mice were fed or injected with xanthurenic acid[4] (m.p. 281–284°, uncorrected). This compound was synthesized according to Musajo and Minchilli (4).

The excreta of groups of four mice or of individual rats were collected and analyzed for xanthurenic acid in the following manner. The urine was collected under a small amount of benzene for 24 hour periods, and the funnels were washed with 5 to 10 cc. of 0.1 per cent $NaHCO_3$ at the end of each period. In most cases each 24 hour sample was analyzed separately, but in some studies pooled samples of 3 to 6 days urine were used. The feces were collected every 24 hours; usually the analyses were made on 3 to 6 day fecal samples from a single rat or 24 hour samples from eight mice. All samples were stored at 5°, and the urine samples were analyzed as described previously (2). The feces were extracted in a mortar with 2 per cent $NaHCO_3$; three extractions of 5 cc. each were used for feces containing normal amounts of chromogen. Feces containing large amounts of xanthurenic acid were extracted with 10 cc. portions until the extract was no longer green (usually 25 to 50 cc.). The combined extracts were centrifuged, and the residue from feces containing xanthurenic acid was reextracted. The combined supernatant solutions were acidified to pH 2.5 ± 0.5, the volume was recorded, and the solution was filtered immediately through qualitative paper. 2 cc. of the filtrate were then extracted in the same manner as urine samples. In the studies on the excretion of administered xanthurenic acid or tryptophane the chromogen was calculated as micrograms of xanthurenic acid excreted per 24 hours in either the feces or urine. In the studies on the activity of certain compounds related to pyridoxine the chromogen was expressed as micrograms of xanthurenic acid excreted in the urine per gm. of food consumed.

The question is still open whether all of the chromogen measured by the techniques employed (extraction from acid with ether-alcohol and color development with $FeCl_3$ in alcohol solution) was xanthurenic acid. Xanthurenic acid has been isolated from the urine of pyridoxine-deficient rats fed high levels of casein (1), and concentrates of the chromogen from mouse urine appeared to be identical in stability, solubility, and optical properties to synthetic xanthurenic acid (2). However, even when large amounts of

[4] We are grateful to Dr. E. E. Snell of the University of Texas for the pyridoxal and pyridoxamine. These compounds are the free bases, so that on a molar basis 0.61 γ of pyridoxal or pyridoxamine is equivalent to 0.75 γ of pyridoxine hydrochloride. The lactone of 2-methyl-3-hydroxy-4-hydroxymethyl-5-carboxypyridine was supplied to Mr. T. D. Luckey and Dr. C. A. Elvehjem by Dr. Karl Folkers of Merck and Company, Inc. We are indebted to Mr. H. E. Säuberlich for aid in the preparation of the xanthurenic acid used in these experiments.

pyridoxine were fed to either rats or mice, a small amount of chromogen was still excreted. The quantity excreted in the urine varied with the casein and tryptophane content of the diet and ranged from 30 to 150 γ per rat or mouse per day. The feces contained 2 to 15 γ per mouse or 10 to 30 γ per rat per day. Since the composition of these fractions is uncertain, these ranges are considered as normal for the purposes of the present discussion, and only larger amounts of chromogen are considered to be xanthurenic acid.

Results

Excretion of Administered Xanthurenic Acid—Musajo and Chiancone (5) isolated xanthurenic acid from the urine of animals fed diets high in tryptophane. Lepkovsky, Roboz, and Haagen-Smit (1) showed that pyridoxine-deficient rats receiving diets high in tryptophane excreted xanthurenic acid, while the addition of pyridoxine to the diet prevented the excretion. Miller and Baumann (2) observed that 10 to 20 per cent of the tryptophane ingested by pyridoxine-deficient mice was excreted in the urine as xanthurenic acid. The deficient mice survived for longer periods on diets of low casein content than on diets high in casein. However, only a part of this effect could be attributed to the tryptophane content of the casein. If xanthurenic acid were toxic to mice, the shorter period of survival on diets high in tryptophane might be due in part to the formation of this compound *in vivo*. Accordingly, a study has been made of the growth and xanthurenic acid excretion of mice fed diets containing xanthurenic acid. The xanthurenic acid excreted by rats was also determined after feeding or injecting single doses.

Weanling albino mice were depleted of pyridoxine on a diet containing 10 per cent of casein and no pyridoxine for 10 to 14 days. Groups of four were then fed 10 per cent casein diets containing 0 or 10 γ of pyridoxine hydrochloride per gm. of diet, with or without xanthurenic acid. In the first series 0.5 mg. of xanthurenic acid was added to each gm. of diet, for pyridoxine-deficient mice have been observed to excrete approximately 0.5 mg. of the acid per gm. of diets containing 30 to 60 per cent of casein (2). The results are indicated in Table I. During an 11 day period 13 to 24 per cent (average 17 per cent) of the ingested xanthurenic acid was excreted in the urine by the mice receiving no pyridoxine, while 14 to 22 per cent (average 19 per cent) was excreted by those receiving 10 γ of pyridoxine per gm. of diet. The mice receiving xanthurenic acid grew as well as those consuming the same diets without xanthurenic acid. Similar results were obtained in a second series when 3 mg. of xanthurenic acid per gm. of diet were fed for 31 days. No differences in growth or survival were observed, and the deficient mice excreted 17 to 20 per cent (average 18 per cent) of the ingested

xanthurenic acid in the urine. Those receiving 10 γ of pyridoxine per gm. of diet excreted 12 to 17 per cent (average 13 per cent) (Table I). Thus, the percentage of ingested xanthurenic acid excreted in the urine by mice did not appear to be influenced significantly by the pyridoxine content of the diet.

Apparently, however, the xanthurenic acid was not completely absorbed. About 10 per cent of the ingested xanthurenic acid could be recovered from the feces, while no appreciable amount of chromogen (equivalent to 2 to 15 γ of xanthurenic acid per day) was present in the feces of mice fed 45 per cent casein diets with either 0 or 10 γ of pyridoxine per gm. The quantity

TABLE I

*Urinary Excretion of Xanthurenic Acid by Mice Given Xanthurenic Acid in Diet**

Expressed as the micrograms of xanthurenic acid consumed or excreted per mouse per 24 hours.

Pyridoxine fed	Days on diet	Control group; xanthurenic acid excreted	Group fed xanthurenic acid			Per cent of ingested xanthurenic acid excreted
			Xanthurenic acid consumed	Xanthurenic acid excreted	Xanthurenic acid excreted above control	
γ per gm. diet		γ	γ	γ	γ	
0	2	65	1,650	306	241	15
	7	162	1,850	467	305	17
10	2	35	1,940	392	357	18
	7	63	1,825	423	360	20
0	2	74	6,080	1175	1101	18
	16	350	8,610	1780	1430	17
10	2	28	8,700	1078	1050	12
	16	55	11,050	1438	1383	13

* The data for the first four readings were obtained with one series of mice; the data for the last four readings, with a second series.

of xanthurenic acid excreted per day in the urine after the ingestion of 3 mg. of xanthurenic acid per gm. of diet was equivalent to that excreted on 45 to 60 per cent casein diets (2), and it is probable that similar amounts were present in the tissues under the two conditions. Yet the mice receiving 3 mg. of the acid per gm. of diet showed no harmful effects over a 31 day period, while similar mice on a 45 per cent casein diet without pyridoxine died in 10 to 20 days (Table IV and (2)).

Reid, Lepkovsky, Bonner, and Tatum (6) made qualitative tests on the urine of rats given single doses of xanthurenic acid. They reported that the xanthurenic acid passed through the pyridoxine-deficient rats unchanged, but could not be recovered from the urine of rats fed pyridoxine. Since the present data with mice are not in harmony with this conclusion,

studies have also been carried out with rats. Weanling rats were fed a 45 per cent casein diet containing no pyridoxine for at least 3 weeks, when the pyridoxine deficiency was established by an excretion of at least 1200 γ of xanthurenic acid per day. They were then fed a 10 per cent casein diet containing no pyridoxine for the duration of the experiment, and the amount of xanthurenic acid excreted on this diet was determined before each test dose was given. After 12 to 20 hours of fasting, 20 mg. of xanthurenic acid in 0.5 gm. of glucose were offered to each rat. When the supplement had been consumed or after 1 hour, the rats were placed in individual metabolism cages and the xanthurenic acid excretion was determined for periods of 3 to 7 days. Other weanling rats were fed a 10 per cent casein diet containing 10 γ of pyridoxine per gm. and single doses of xanthurenic acid in the same manner. Under these conditions 11 to 30 per cent of the xanthurenic acid ingested (12 to 20 mg.) was excreted in the urine within 1 week by both pyridoxine-deficient and pyridoxine-fed rats. Of this 60 to 80 per cent was excreted in the first 2 days, and no more than 14 per cent was excreted in the last 4 days. However, rats, like mice, appeared to absorb xanthurenic acid poorly. 5 to 25 per cent of the dose was excreted in the feces, and the combined fecal and urinary excretion was 20 to 40 per cent. When 20 mg. of *l*-tryptophane were fed in this manner to pyridoxine-deficient rats, about 15 per cent was excreted in the urine as xanthurenic acid and only normal amounts of chromogen were found in the feces.

Other rats treated in the same manner, but not fasted, were injected intraperitoneally with 14.3 mg. of xanthurenic acid in 1 cc. of 2 per cent $NaHCO_3$. 50 per cent (44 to 54) of the xanthurenic acid was excreted in the urine by the deficient rats in the following 24 hours, while the rats fed pyridoxine excreted 58 per cent (50 to 62) (Table II). No more than 2 per cent of the dose was excreted in the second 24 hour period, and the excretion was nearly normal by the 3rd day. 0 to 4 per cent was found in the feces. When 14.3 mg. of *l*-tryptophane were injected intraperitoneally into the same rats 1 week later, those deficient in pyridoxine excreted 10 to 12 per cent of the dose as xanthurenic acid during the first 24 hours, while the rats receiving pyridoxine excreted only 0.5 to 1 per cent (Table II). After 3 days the total urinary excretion was 12 to 16 per cent for the deficient rats and about 1 per cent for those fed pyridoxine.

Thus, both rats and mice excreted a part of administered xanthurenic acid in the urine, and in all cases the state of pyridoxine nutrition of the animals had no effect on the amount excreted. On the other hand, pyridoxine-deficient animals excreted 10 to 20 per cent of administered *l*-tryptophane (2) as xanthurenic acid, while similar animals fed pyridoxine excreted no more than 1 per cent. These facts suggest that xanthurenic acid may not be a normal metabolite of tryptophane for either species; for if it were,

those receiving adequate pyridoxine should have been able to metabolize the administered xanthurenic acid more effectively than the deficient animals. The amount excreted would then have approached that found after feeding tryptophane with adequate pyridoxine. It would seem therefore that one function of pyridoxine is to regulate the metabolism of tryptophane by a route that prevents the formation of appreciable amounts of xanthurenic acid. Further, xanthurenic acid was found in the feces only after feeding the compound itself, and not after feeding either high casein diets or single doses of tryptophane. It appears, therefore, that the xanthurenic

TABLE II

Urinary Excretion of Xanthurenic Acid by Rats Injected Intraperitoneally with Xanthurenic Acid or l-Tryptophane

Rat No.	Pyridoxine fed	Xanthurenic acid injected	Tryptophane injected	Xanthurenic acid excreted			
				Basal period	Following injection	Excess above basal	Per cent of dose*
	γ per gm.	γ	γ	γ	γ	γ	
1	0	14,300		142	7780	7638	53
2	0	14,300		247	8000	7753	54
3	10	14,300		39	7180	7141	50
4	10	14,300		65	8900	8835	62
1	0		14,300	80	1490	1410	10
2	0		14,300	200	1950	1750	12
3	10		14,300	110	238	128	1
4	10		14,300	100	183	83	<1

* These figures are for the first 24 hours after injection. Approximately 2 per cent more of the injected xanthurenic acid was excreted by each of the rats in the following 24 hours; 2 to 4 per cent more of the injected tryptophane was excreted as xanthurenic acid in the subsequent 48 hours by the pyridoxine-deficient rats, but no more was excreted by the control rats.

acid is formed from the tryptophane principally in the animal body rather than in the intestinal tract.

Relative Activity of Pyridoxal, Pyridoxamine, Pyridoxine, and Lactone of 5-Carboxypyridoxine—Pyridoxal and pyridoxamine have been synthesized by Harris, Heyl, and Folkers (7) and may occur in natural products (8). These compounds have been reported to be much more active than pyridoxine for certain microorganisms (9) and to have equal or greater activity for rats (9). Pyridoxal is necessary for the tyrosine decarboxylase activity of *Streptococcus faecalis* R (10), and may be of importance in transamination (11, 12). The substitution of pyridoxamine or pyridoxal for pyridoxine in the media of *Lactobacillus casei* or *Lactobacillus delbrückii* permits maximum growth in the absence of lysine, threonine, and alanine, although these

amino acids are necessary for appreciable growth when pyridoxine is used (13). We have extended these comparative studies by testing the activity of pyridoxal and pyridoxamine for mice and rats, with growth and the level of xanthurenic acid excretion as criteria of vitamin potency.

Weanling albino mice were depleted of pyridoxine on a 10 per cent casein diet for 14 days, and then fed 45 per cent casein diets containing the following supplements per gm.: no supplement, 0.75 or 3.0 γ of pyridoxine hydrochloride, 0.61 or 2.44 γ of pyridoxal, or 0.61 or 2.44 γ of pyridoxamine. Thus the three compounds were fed at two levels in equivalent molar quantities. While 3 γ of pyridoxine hydrochloride brought the xanthurenic acid excretion close to the normal range in 4 days, 17 days were required with an equivalent quantity of pyridoxal and 24 days with pyridoxamine (Table III). The mice receiving 2.44 γ of pyridoxal grew at a slower rate than those receiving pyridoxine for 1 week, after which the growth rates

TABLE III

Xanthurenic Acid Excretion and Growth of Mice Fed Diets Containing 45 Per Cent of Casein with Pyridoxine Hydrochloride, Pyridoxal, or Pyridoxamine (Four Mice Per Group)

Supplement	None	0.75 γ pyridoxine hydrochloride	0.61 γ pyridoxal*	0.61 γ pyridoxamine	3.0 γ pyridoxine hydrochloride	2.44 γ pyridoxal	2.44 γ pyridoxamine
Xanthurenic acid excreted in urine per gm. diet consumed							
	γ	γ	γ	γ	γ	γ	γ
Basal period†	117	145	73	133	92	100	90
4th day	370	710	638	289	83	158	331
11th "	590	507	305		40	137	369
17th "		528			45	56	131
24th "		1060			45	27	78
Average weight							
	gm.	gm.	gm.	gm.	gm.	gm.	gm.
1st day	9.5	9.3	9.7	10.8	9.7	9.7	9.4
7th "	8.3	8.6	8.9	9.6	10.9	10.1	9.5
17th "		8.6			12.9	13.2	10.4
31st "		9.2			16.4	14.5	12.1
Survival in days	12–16	>31	13–17	6–10	>31	>31	>31

* On a molar basis 0.61 γ of pyridoxal or pyridoxamine is equivalent to 0.75 γ of pyridoxine hydrochloride; 2.44 γ are equivalent to 3.0 γ of pyridoxine hydrochloride.

† The figures for the basal period were obtained on the last day of the 14 day basal period during which the mice received 10 per cent of casein and no pyridoxine.

were nearly equal. However, the mice receiving 2.44 γ of pyridoxamine grew poorly throughout the experiment. By the 17th day the mice receiving 3 γ of pyridoxine hydrochloride had gained 3.2 gm., while those receiving equivalent amounts of pyridoxal or pyridoxamine had gained 3.5 and 1.0 gm., respectively. When the lower levels of these compounds were fed, the difference was largely one of survival. The mice receiving no pyridoxine died in 14 days (12 to 16), and those receiving 0.75 γ of pyridoxine hydrochloride survived for the duration of the experiment (31 days). On the other hand, the mice receiving 0.61 γ of pyridoxal or pyridoxamine died in 15 days (13 to 17) and 9 days (6 to 10), respectively. The comparative excretion of xanthurenic acid by these mice was not considered significant, since such values tend to be lower and more variable for a few days before death.

This experiment was repeated with mice that had been depleted for only 10 days. In the second series the xanthurenic acid excretion of the mice receiving the high levels of pyridoxal or pyridoxamine fell to the normal range after 12 days of administration, while only 2 days were required for mice receiving equivalent pyridoxine. Though the mice receiving 2.44 γ of pyridoxal or pyridoxamine gained only slowly for the first few days, by the 12th day their gain (3.2 gm.) equaled that of the pyridoxine-fed mice. Of the mice receiving the lower levels of these compounds the most xanthurenic acid was excreted by those on pyridoxamine and the least by those supplemented with pyridoxine. After 19 days all of the mice receiving no supplement were dead, while one of the four mice on the low level of pyridoxamine had died. At this time the mice receiving 0.75 γ of pyridoxine hydrochloride had gained 3.8 gm., while those receiving pyridoxal or pyridoxamine had lost 1.1 and 0.8 gm., respectively. Thus, as determined both by their ability to promote growth and to prevent the excretion of xanthurenic acid on diets high in casein, pyridoxamine and pyridoxal appear to be less active for mice than pyridoxine.

Since no data were available on the stability of these compounds in rations stored at 5°, we analyzed the diets for their pyridoxine activity. 5 gm. of each diet were extracted at room temperature once with 100 cc. and twice with 30 cc. of 0.05 N HCl. The extracts were filtered, and the residue washed twice with 15 cc. of the acid. The combined extracts and washings were then neutralized to pH 4.7, filtered, adjusted to pH 5.2, and diluted to contain 15 millimicrograms of pyridoxine hydrochloride or its equivalent per cc. The samples were analyzed by the growth of *Saccharomyces carlsbergensis* according to Atkin *et al.* (14), except that the medium and samples were sterilized separately. Standard solutions of pyridoxal and pyridoxamine showed activities equal (0.95 to 1.05 times as active) to that of pyridoxine. 2 months after mixing, 94 to 100 per cent of

the supplements was still present (Table IV). Accordingly, the effects observed could not have been due to the destruction of the vitamins in the diets.

In contrast to the lower activities of pyridoxal and pyridoxamine for mice, Snell and Rannefeld (9) have reported that on a weight basis pyridoxal was 1.2 times as active for white rats as pyridoxine hydrochloride, while pyridoxamine was 1.6 times as active. On a molar basis their ratios would be 1:1:1.3 for pyridoxine, pyridoxal, and pyridoxamine, respectively. Under conditions similar to those used for the experiments with mice, these compounds also appeared to be less active for rats than pyridoxine. Weanling female Sprague-Dawley rats were depleted for 2 weeks on a 45 per cent

TABLE IV

Stability of Pyridoxal, Pyridoxamine, and Pyridoxine Hydrochloride in Synthetic Diets (2 Months Storage at 5°)

Supplement added to diet			Quantity found by analysis*	
Compound	Amount	Expressed as pyridoxine hydrochloride	Expressed as pyridoxine hydrochloride	Per cent recovery
	γ *per gm.*	γ *per gm.*	γ *per gm.*	
Pyridoxine hydrochloride	0.75	0.75	0.75	100
Pyridoxal	0.61	0.75	0.73	97
Pyridoxamine	0.61	0.75	0.72	96
Pyridoxine hydrochloride	3.00	3.00	2.80	94
Pyridoxal	2.44	3.00	2.80	94
Pyridoxamine	2.44	3.00	2.90	97

* Determined by the growth of *Saccharomyces carlsbergensis* according to Atkin *et al.* (14). Pyridoxal, pyridoxamine, and pyridoxine hydrochloride were found to have equal activity on a molar basis.

casein diet. Groups of four were then fed 45 per cent casein diets containing either no supplement, 0.5 γ of pyridoxine hydrochloride, 0.41 γ of pyridoxal, or 0.41 γ of pyridoxamine per gm. (molar equivalents). Over a 4 week period the average gains per week were 2, 8, 5, and 6 gm. for the basal group and those supplemented with pyridoxine, pyridoxal, or pyridoxamine, respectively. When this experiment was repeated with 0.63 γ of pyridoxine hydrochloride and 0.51 γ of pyridoxal or pyridoxamine per gm. of diet, the average weekly gains were 3, 13, 8, and 9 gm., respectively. The supplements were fed at these levels, since Conger and Elvehjem (15) have shown that the growth of rats previously depleted of pyridoxine was approximately a linear function of the administered pyridoxine at levels of 2 to 6 γ per day.

It is not clear why Snell and Rannefeld (9) found pyridoxal and pyridoxamine to have activity equal to or greater than pyridoxine for rats, while our

data indicate that they are less active; however, differences in procedure might be responsible. Snell and Rannefeld (9) fed male rats a basal diet (15) containing 18 per cent of casein, and the daily supplement was administered as a single dose. We fed a diet containing 45 per cent of casein, and the supplements were added to the diets in equimolar concentrations. Only female rats were used. It is possible that pyridoxal or pyridoxamine might be oxidized more readily than pyridoxine to 4-pyridoxic acid in the body. This compound has been isolated from urine after the ingestion of pyridoxine (16), and it is inactive for rats (9).

Since the lactone of 2-methyl-3-hydroxy-4-hydroxymethyl-5-carboxypyridine has been reported to have antianemic properties for chicks (17) and is related structurally to pyridoxine, we tested the activity of this compound for pyridoxine-deficient mice. Weanling mice depleted of pyridoxine for 8 days on the 10 per cent casein diet were fed 45 per cent casein diets containing either no supplement, 0.75, or 3.0 γ of pyridoxine hydrochloride, or 2.4 or 8.0 γ of the lactone per gm. of diet. On a molar basis these amounts of lactone are equivalent to 3 and 10 γ of pyridoxine hydrochloride. The mice receiving no pyridoxine died in 9 days, while those receiving 0.75 or 3.0 γ of pyridoxine hydrochloride all survived for the duration of the experiment (21 days). The mice receiving the low level of the lactone lived for only 11 days, while those receiving the high level all survived for at least 18 days. By the 15th day the mice receiving 8.0 γ of the lactone had lost 2 gm., while those receiving 0.75 or 3.0 γ of pyridoxine hydrochloride had gained 1.0 and 3.7 gm., respectively. As judged by survival, the lactone appeared to have slight pyridoxine activity. However, it is also possible that small amounts of pyridoxine formed during the preparation of the lactone (18) might have been present in the sample used.

SUMMARY

1. Mice fed 0.5 or 3.0 mg. of xanthurenic acid per gm. of diet excreted about 16 per cent of the acid in the urine. Rats fed single doses (12 to 20 mg.) excreted 10 to 30 per cent in the urine in 3 to 7 days, while those injected intraperitoneally excreted 50 to 58 per cent of the acid within 24 hours. The percentage of the doses that could be recovered was essentially the same whether pyridoxine was administered or not.

2. After the intraperitoneal injection of *l*-tryptophane, pyridoxine-deficient rats excreted 12 to 16 per cent as xanthurenic acid in the urine, while only 1 per cent was excreted by rats receiving pyridoxine in the diet.

3. Mice fed xanthurenic acid in the diet excreted about 10 per cent of the compound in the feces; rats fed single doses excreted 5 to 25 per cent in the feces. No increased fecal excretion was observed after feeding diets high

in casein to pyridoxine-deficient or control rats and mice, or after feeding single doses of tryptophane to pyridoxine-deficient rats.

4. The consumption of 3 mg. of xanthurenic acid per gm. of diet for 31 days had no effect on the growth or survival of control or pyridoxine-deficient mice, although these mice excreted as much xanthurenic acid in the urine as did deficient mice on 45 per cent casein diets.

5. As judged by both growth and excretion of xanthurenic acid, pyridoxamine and pyridoxal were less active for mice than pyridoxine. By growth tests these compounds also appeared to be less active for rats. A sample of the lactone of 2-methyl-3-hydroxy-4-hydroxymethyl-5-carboxypyridine had only slight activity for mice.

BIBLIOGRAPHY

1. Lepkovsky, S., Roboz, E., and Haagen-Smit, A. J., *J. Biol. Chem.*, **149**, 195 (1943).
2. Miller, E. C., and Baumann, C. A., *J. Biol. Chem.*, **157**, 551 (1945).
3. Wesson, L. G., *Science*, **75**, 334 (1932).
4. Musajo, L., and Minchilli, M., *Ber. chem. Ges.*, **74 B**, 1839 (1941).
5. Musajo, L., and Chiancone, F. M., *Gazz. chim. ital.*, **67**, 218 (1937).
6. Reid, D. F., Lepkovsky, S., Bonner, D., and Tatum, E. L., *J. Biol. Chem.*, **155**, 299 (1944).
7. Harris, S. A., Heyl, D., and Folkers, K., *J. Am. Chem. Soc.*, **66**, 2088 (1944).
8. Snell, E. E., *J. Biol. Chem.*, **157**, 491 (1945).
9. Snell, E. E., and Rannefeld, A. M., *J. Biol. Chem.*, **157**, 475 (1945).
10. Gunsalus, I. C., and Bellamy, W. D., *J. Biol. Chem.*, **155**, 357 (1944).
11. Schlenk, F., and Snell, E. E., *J. Biol. Chem.*, **157**, 425 (1945).
12. Snell, E. E., *J. Am. Chem. Soc.*, **67**, 194 (1945).
13. Stokes, J. L., and Gunness, M., *Science*, **101**, 43 (1945).
14. Atkin, L., Schultz, A. S., Williams, W. L., and Frey, C. N., *Ind. and Eng. Chem., Anal. Ed.*, **15**, 141 (1943).
15. Conger, T. W., and Elvehjem, C. A., *J. Biol. Chem.*, **138**, 555 (1941).
16. Huff, J. W., and Perlzweig, W. A., *J. Biol. Chem.*, **155**, 345 (1944).
17. Scott, M. L., Norris, L. C., Heuser, G. F., Bruce, W. F., Coover, H. W., Jr., Bellamy, W. D., and Gunsalus, I. C., *J. Biol. Chem.*, **154**, 713 (1944).
18. Harris, S. A., Stiller, E. T., and Folkers, K., *J. Am. Chem. Soc.*, **61**, 1242 (1939).

THE COLORIMETRIC DETERMINATION OF ARGININE IN PROTEIN HYDROLYSATES AND HUMAN URINE*

By ANTHONY A. ALBANESE† and JANE E. FRANKSTON

(*From the Department of Pediatrics, The Johns Hopkins University and the Harriet Lane Home of the Johns Hopkins Hospital, Baltimore*)

(Received for publication, March 12, 1945)

The colorimetric estimation of arginine as described by Sakaguchi (1) is based on the unstable red color produced by the addition of α-naphthol and 0.3 N sodium hypochlorite to an alkaline solution of the amino acid. In 1930 Weber (2) and subsequently other investigators (3–7) reported that greater color stability could be attained by the substitution of 0.3 N sodium hypobromite for the hypochlorite, by providing for the destruction of excess hypohalite by the addition of urea and carrying out the test at 4°. A careful study of the various modifications of the original Sakaguchi reaction led us to the finding that the difficulties arising from the use of sodium hypochlorite are not due to any particular property of the reagent as suggested by Weber but are rather a fault of the concentration recommended by Sakaguchi. In our experiments we have found that the use of 0.06 N sodium hypochlorite in conjunction with urea results in a color stability greater than that obtainable with 0.3 N or 0.06 N sodium hypobromite. Our studies have further shown that the use of hypochlorite affords the following technical advantages: (*a*) it permits the performance of the test at room temperatures (20–25°) without detectable loss of color stability or intensity, (*b*) it obviates some of the troublesome adjustment of reagent quantities to the nitrogen content or composition of the sample so necessary with hypobromite, and (*c*) it yields relatively low reagent blank readings.

The method developed on the basis of these experiments has been applied to a number of proteins and has been found to yield results which are in fair accord with those obtained by other investigators. In an attempt to apply the procedure to urine the interference arising from other chromogens and the possible presence of glycocyamine and methylguanidine had to be considered and indicated the use of permutit for the separation of arginine from the sample (8). However, our inability to elute the

* The work described in this report was supported by grants from the Rockefeller Foundation and the Nutrition Foundation, Inc. Partial support was also derived under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and The Johns Hopkins University.

† Present address, Children's Medical Service, Bellevue Hospital, New York.

adsorbed arginine quantitatively from the permutit by the use of various reagents, in the limited quantities permitted by the test conditions, led to the adoption of the device of estimating urinary arginine as the difference of the red color values of an aliquot treated with permutit and one not so treated. This scheme circumvented all of the apparent difficulties except the one contingent on the possible presence of methylguanidine, which seems to be unavoidable. The adequacy of this procedure for analytical purposes was demonstrated by recovery tests. The analyses of 24 hour specimens of thirty-seven normal adult males (60 to 100 kilos) on normal diets have shown the daily arginine output to fall between 50 and 150 mg., which represents but 1 to 2 per cent of the total amino N. However, chemical as well as feeding tests failed to permit of an estimation of the amount of monomethylguanidine which is included in this urinary arginine measurement.

EXPERIMENTAL

Reagents—

Sodium hypochlorite. A 0.06 N solution was prepared as needed from the commercially available Clorox. The necessary dilution of the stock product was ascertained iodometrically as follows: To 1 cc. of Clorox in a 125 cc. Erlenmeyer flask are added 25 cc. of chlorine-free water in which has been dissolved 1 gm. of KI. The mixture was then titrated with 0.1 N sodium thiosulfate, 1 cc. of starch indicator being used. The stock product has been found to be fairly stable for 3 to 4 months if kept in the refrigerator in a brown bottle.

Sodium hydroxide, 10 per cent solution.

Urea, 20 per cent solution.

Permutit. A 60 mesh product was used and was activated as described by Whitehorn (8).

α-Naphthol. 100 mg. of the resublimed product are dissolved in 100 cc. of 95 per cent ethanol. The solution is kept in a brown bottle and stored in the refrigerator.

Arginine standard. 12.05 mg. of $l(+)$-arginine hydrochloride, Merck (26.6 per cent N found), are weighed accurately and dissolved in 100 cc. of water. 1 cc. of this solution is equivalent to 100 γ of the free base. This solution is stored in the refrigerator.

Analytical Procedure

Hydrolysates were prepared by refluxing 5.0 gm. samples of the proteins with 25 cc. of constant boiling (6 N) hydrochloric acid for 24 hours. The total nitrogen content of the hydrolysate is determined directly by micro-Kjeldahl analysis (9); the excess of acid is then removed by concentration

in vacuo and the humin separated by filtration. Sulfuric acid digests are prepared by refluxing 5.0 gm. of protein with 25 cc. of 25 per cent (by weight) sulfuric acid (6 N) for 24 hours and removing the acid as calcium sulfate. These amounts of protein are of course far in excess of the 0.5 to 1.0 mg. of hydrolysate nitrogen actually required for the arginine determination.

Aliquots of these hydrolysates not greater than 5 cc. in volume and containing 100 to 400 γ of arginine are measured into 10 cc. graduated Klett-Summerson photoelectric colorimeter tubes. If necessary, the volume of the sample is adjusted to 5 cc.; 1 cc. of 10 per cent NaOH and 1 cc. of α-naphthol reagent are then added with mixing. After 5 minutes, 1 cc. of sodium hypochlorite is added, followed exactly 1 minute later by the addition of 2 cc. of 20 per cent urea solution. The resulting solutions are thoroughly mixed by inverting the tubes and read in the Klett-Summerson colorimeter with Filter S-54. The color intensity of the reaction mixture has been found to remain constant for more than 15 minutes, so that there is no need to hasten the color measurement. A parallel determination is also done on an aliquot of the standard containing an amount of arginine comparable to that of the unknown.

Urine—24 hour specimens are collected in brown bottles containing 50 cc. of 15 per cent HCl (by volume) and 1 cc. of 10 per cent alcoholic thymol and are made to a uniform volume of 2 liters before the removal of the samples for the determination. The arginine content of the specimens was found not to change after a storage of 10 days at room temperature.

In order to overcome the interference from glycocyamine and the intense yellow coloration which forms on treating urine with the reagents required for the arginine determination, urinary arginine was estimated as the difference in color intensity obtained by performing the test on aliquots of the urine sample before and after treatment with permutit. This scheme was adopted after numerous experiments showed that not more than 50 per cent of the arginine adsorbed on permutit could be recovered by the use of the limited quantities of 3 per cent NaCl (7), 25 per cent KCl (8), or 10 per cent NaOH (10) permitted by the conditions of the test. On the basis of these experiments the following procedure was developed which has proved convenient for routine purpose and is capable of highly reproducible results. 20 to 25 cc. of urine (pH 5 to 6) are passed at the rate of 1 drop per second through a column of 10 gm. of activated permutit contained in a 150 × 15 mm. calcium chloride tube plugged with coarse glass wool and fitted with a short piece of rubber tubing and pinch-cock to regulate the flow. 5 cc. of this filtrate and 5 cc. of the original urine sample are each transferred to 10 cc. graduated colorimeter tubes and treated as

already described for proteins. The difference in the readings of such a pair of tests represents the arginine content of 5 cc. of urine.

Calculations—

A = colorimetric reading of protein sample
U = " " " 5 cc. urine sample
P = " " " 5 " permutit filtrate
B = " " " arginine standard
C = " " " reagent blank
$A' = A - C$, corrected colorimetric reading of protein sample
$U' = U - C$, " " " " urine sample
$P' = P - C$, " " " " permutit filtrate
$B' = B - C$, " " " " arginine standard

Then for *proteins*, mg. of arginine in sample = $(A'/B') \times$ mg. of arginine in standard; for *urine*, mg. of arginine in 24 hour specimen = $((U' - P')/B') \times$ mg. of arginine in standard (total volume of 24 hour specimen)/5.

Results

In order to ascertain the analytical efficacy of the modifications made in the conditions of the original Sakaguchi reaction suitable aliquots of the arginine standard were submitted to the procedure previously outlined for protein hydrolysates. The linear relationship of the color intensity to the amount of arginine which appears to exist (Fig. 1) is interpreted as evidence of the adequacy of the modifications and of the validity of Beer's law for the color reaction. This relationship was observed to prevail without appreciable deviation over the range 6–26°, but became erratic above this temperature.

The results of experiments designed to test the relative stability of colors obtained by the use of different concentrations of hypochlorite and hypobromite are shown in Fig. 2. The relatively high color stability and low reagent blank readings obtained with 0.06 N NaOCl clearly recommend the employment of this reagent in the quantitative application of the Sakaguchi reaction. Loss of color stability and high reagent blank readings resulted when (*a*) 0.01 N instead of 0.06 N sodium hypochlorite was used, or (*b*) the urea reagent was added to the reaction mixture before 0.06 N sodium hypochlorite (6), or (*c*) the urea addition was omitted.

The effect of variation in nitrogen content and composition of the sample on the arginine color value was also tested and the relevant data are shown in Fig. 3. It is evident from these experiments that the use of dilute hypochlorite eliminates to a large extent the adjustments of reagent quantities to the sample characteristics which seem so necessary when 0.3 N sodium hypochlorite (1) and 0.3 N sodium hypobromite (5) are employed. It is to be noted, however, that a marked drop in the normal colorimeter readings of the arginine standard (0.20 mg.) was sustained when it was

tested in the presence of 10-fold quantities of histidine and ammonium sulfate, a condition which is seldom attained in biological products.

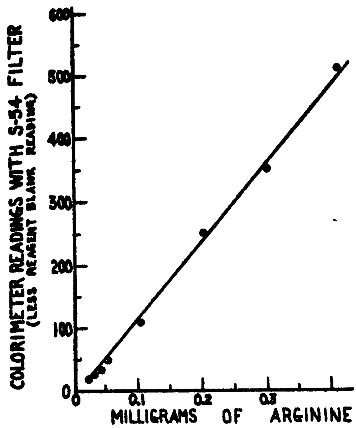


FIG. 1. Relation of color intensity to amount of arginine. Each area represents the average value and average deviation of ten or more determinations performed at 20–25°.

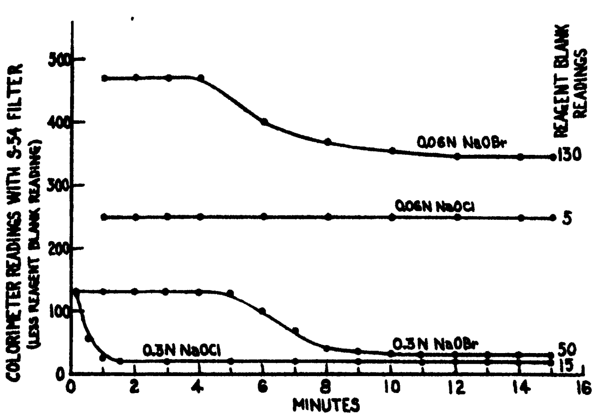


FIG. 2. Effect of varying the concentration of sodium hypochlorite or hypobromite on the color intensity and stability of the Sakaguchi reaction. 0.21 mg. of arginine contained in 5 cc. of water was used in each test. The reactions were all performed at 23°, with 1 cc. of 10 per cent NaOH, 1 cc. of 0.1 per cent α-naphthol in 95 per cent ethanol, 1 cc. of hypohalite reagent, and 2 cc. of 20 per cent urea solution, making a final volume of 10 cc.

The applicability of the modified reaction to the estimation of arginine in protein hydrolysate and the urine was further assayed by means of recovery tests (Table I). The quantitative recovery of arginine added to

different amounts of hydrolysate nitrogen supplements the evidence presented below as to the relative lack of sensitivity of the modified reaction in respect to variations in the quantity and quality of the sample nitrogen.

The results of the analyses of some protein hydrolysates are given in Table II. Most of the analyses were performed on hydrolysates prepared with hydrochloric acid. Commercially available proteins, casein, lactalbumin, gelatin, and cattle fibrin were used without further refinement of the products. However, the moisture and ash contents of these products were determined and the analytical results corrected for these impurities. Human hemoglobin was derived from red blood cells by the procedure of Zinoffsky (11). A pancreatic digest of casein containing 32.3 per cent of the total N as free amino N was submitted to the analysis without further hydrolysis. Attention is called to the remarkably slight differences in arginine

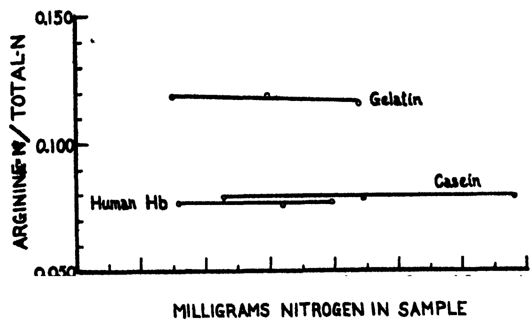


FIG. 3. Effect of differences of sample nitrogen on the arginine N to total N relationship of various proteins. These determinations were all performed as described in the text.

values found for the variously prepared casein digests. Also, the values obtained for casein, fibrin, and gelatin compare favorably with those previously reported by one of us (12) as well as by others (Table III).

Although the quantitative recovery of arginine added to urine (Table I) points to adequacy of the procedure for the determination of arginine in the urine, it was considered advisable to secure evidence for the specificity of the reaction from excretion tests. To this end fasting normal subjects were fed 2.10 gm. (0.01 M) of $l(+)$-arginine + HCl 2 hours after breakfast and given 240 cc. of water at zero hour and 120 cc. more at the end of each of the next 4 hours to maintain a uniform flow of urine for the experimental period. The urine was collected hourly for 5 hours and analyzed immediately for arginine, creatine, and creatinine (16) and for urea (17). From the typical data shown in Fig. 4 it is apparent that the major portion of the ingested arginine is excreted as urea and produces only a slight eleva-

TABLE I

Recovery of Arginine Added to Protein Hydrolysates and Urine

Biological product	Total N	Arginine added	Arginine content	Recovery of added arginine
	mg.	γ	γ	*per cent*
Casein, Harris (HCl digest)	0.660		169	
	0.660	103	273	100.5
	0.330		84	
	0.330	103	186	99.0
Gelatin, U. S. P. (HCl digest)	0.543		262	
	0.543	103	365	100.0
	0.271		131	
	0.271	103	232	98.0
5 cc. Urine A	0.345		45	
	0.345	27	73	101.0

TABLE II

Arginine Content of Hydrolysates of Some Biological Substances

Biological product	Hydrolyzing agent	Uncorrected N content	Moisture content	Ash content	Corrected N	Arginine N of total N
		per cent	*per cent*	*per cent*	*per cent*	*per cent*
Casein, Harris..........	HCl	13.62	6.80	0.38	14.63	7.93
Lactalbumin, Harris....	"	11.70	7.00	1.92	12.71	6.00
Gelatin, U. S. P.........	"	14.30	12.10	0.42	16.09	11.82
Cattle fibrin, Wilson....	"	15.10	4.03	0.21	15.70	13.95
Casein, Sheffield.........	H_2SO_4	12.50				7.70
" "	Pancreatin*	12.50				7.74
Human hemoglobin.....	HCl	13.96				7.70

* Kindly supplied by the Takamine Laboratory, Inc.

TABLE III

Comparison of Results of Arginine Analyses of Proteins by Modified Sakaguchi Reaction and Other Methods

Investigators	Method	Arginine N in per cent of total N		
		Gelatin	Cattle fibrin	Casein
Present..........................	Colorimetric	11.82	13.95	7.93
Albanese (12)....................	Electrolytic	11.62	13.98	7.35
Van Slyke (13)...................	Van Slyke	14.70	13.2–14.3	7.40
Hunter and Dauphinee (14)	Arginase	15.68	14.31	7.95
Vickery (15).....................	Diflavianate	15.30	14.80	7.70

tion in the arginine output and no rise in the creatine and creatinine levels (18). The evidence derived from these experiments suggests that our test

is a measure of arginine, but the possibility that it may also include or be due to methylguanidine must be borne in mind and will be discussed later.

Results of the arginine analysis of 24 hour urine specimens of seven normal adult males on normal diets are given in Table IV. The amino

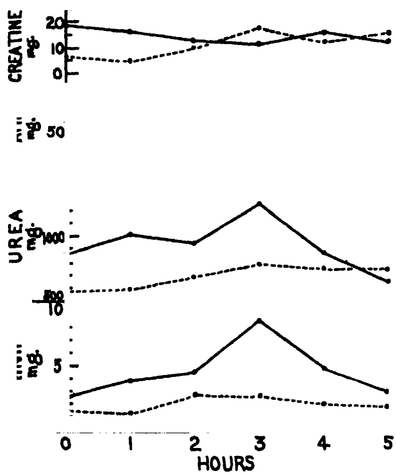


FIG. 4. Urinary output of arginine and other metabolites of a fasting subject, A, male, after administration of 0.01 mole (2.1 gm.) of $l(+)$-arginine + HCl. The solid lines indicate excretion after ingestion of arginine and the broken lines normal urinary output.

TABLE IV

Relation of Arginine Excretion to Total N and Amino N Output in Normal Adult Male

Subject	Body weight	Total N	Amino N	Arginine	Arginine amino N of amino N
	kg.	*gm.*	*mg.*	*mg.*	*per cent*
Br	103.0	19.4	596	60	0.87
Vo	94.0	17.4	696	60	0.69
Ba	74.4	15.8	459	108	1.94
Yo	81.6	10.8	392	68	1.39
So	90.1	14.3	493	72	1.17
Me	80.7	15.5	408	36	0.71
Sh	68.0	13.8	459	70	1.23

N (19) and total N (9) contents of these specimens were also determined and it appears from these data that arginine amino N constitutes from 1 to 2 per cent of the total amino N. These values are representative of those obtained from the study of thirty other normal adult males. It is sig-

nificant that the arginine output of eight subjects whose urine was examined for 60 consecutive days was found to exhibit considerable individual diurnal variation.

Comments

The experiments reported here indicate that the use of 0.06 N NaOCl instead of 0.3 N NaOBr in the Weber modification of the Sakaguchi reaction results in greater color stability and intensity. The greater accuracy and convenience of operation which arise from these factors and the findings that our modification of the reaction can be performed at room temperatures without the careful adjustment of reagent quantities employed according to the concentration of arginine in the sample recommend the method for studies requiring numerous arginine determinations in proteins or urines.

In considering the significance of the urinary arginine measurement it must be borne in mind that of the ten guanidine derivatives shown by Poller (20) to react positively in the Sakaguchi test only three, arginine (21), glycocyamine (22), and monomethylguanidine (23), are definitely known to occur in human urine. Inasmuch as glycocyamine is not adsorbed by permutit (7), a positive test in the urine by any procedure based on the Sakaguchi reaction would indicate the presence of either arginine or monomethylguanidine or both. The similarity of these two compounds in this and other chemical characteristics makes their separation most difficult even by isolation techniques. Although our feeding experiments show that some dietary arginine may be excreted as such, they do not preclude the possibility that the measurement does not include some methylguanidine which might be derived metabolically from the ingested arginine. Attempts in this experiment to estimate by the arginase reaction (14) what proportion of the urinary arginine as measured by the Sakaguchi test was really arginine failed owing to technical difficulties. In this connection, however, it is important to note that Mueller (24) was able to isolate appreciable quantities of arginine from the urine of rabbits fed unusually large amounts of arginine carbonate.

The effect of the ingestion of racemic arginine monohydrochloride on the output of arginine and other metabolites is under investigation.

SUMMARY

It has been found that the substitution of 0.06 N sodium hypochlorite for 0.3 N sodium hypobromite in the Weber modification of the Sakaguchi reaction affords greater convenience of operation and accuracy of the determination of arginine in protein hydrolysates and human urine. The arginine values for protein hydrolysates obtained by this method are com-

pared with those secured by other techniques. The urinary analyses of 24 hour specimens from thirty-seven normal adult males indicate that 50 to 150 mg. of arginine are excreted daily. The possibility that a portion or all of this measurement may be due to the presence of methylguanidine is discussed.

We wish to thank Miss Virginia Irby for some determinations performed in connection with this work.

BIBLIOGRAPHY

1. Sakaguchi, S., *J. Biochem.*, Japan, **5**, 133 (1925).
2. Weber, C. J., *J. Biol. Chem.*, **86**, 217 (1930).
3. Jorpes, E., and Thoren, S., *Biochem. J.*, **26**, 1504 (1932).
4. Thomas, L. E., Ingalls, J. K., and Luck, J. M., *J. Biol. Chem.*, **129**, 263 (1939).
5. Brand, E., and Kassell, B., *J. Biol. Chem.*, **145**, 359 (1942).
6. Macpherson, H. T., *Biochem. J.*, **36**, 59 (1942).
7. Dubnoff, J. W., *J. Biol. Chem.*, **141**, 711 (1941). Dubnoff, J. W., and Borsook, H., *J. Biol. Chem.*, **138**, 381 (1941).
8. Whitehorn, J. C., *J. Biol. Chem.*, **56**, 751 (1923).
9. Meeker, E. W., and Wagner, E. C., *Ind. and Eng. Chem., Anal. Ed.*, **5**, 396 (1933).
10. Folin, O., *J. Biol. Chem.*, **51**, 377, 393 (1922).
11. Zinoffsky, C., *Z. physiol. Chem.*, **10**, 16 (1886).
12. Albanese, A. A., *J. Biol. Chem.*, **134**, 467 (1940).
13. Van Slyke, D. D., *J. Biol. Chem.*, **10**, 15 (1911–12); **16**, 531 (1913–14).
14. Hunter, A., and Dauphinee, J. A., *J. Biol. Chem.*, **85**, 627 (1929–30).
15. Vickery, H. B., *J. Biol. Chem.*, **132**, 325 (1940).
16. Albanese, A. A., and Wangerin, D. M., *Science*, **100**, 2586 (1944).
17. Barker, S. B., *J. Biol. Chem.*, **152**, 453 (1944).
18. Hyde, E. C., and Rose, W. C., *J. Biol. Chem.*, **84**, 535 (1929).
19. Albanese, A. A., and Irby, V., *J. Biol. Chem.*, **153**, 583 (1944).
20. Poller, K., *Ber. chem. Ges.*, **59**, 1927 (1926).
21. Wohlgemuth, J., *Z. physiol. Chem.*, **44**, 74 (1904).
22. Weber, C. J., *Proc. Soc. Exp. Biol. and Med.*, **32**, 172 (1934); *Proc. Am. Soc. Biol. Chem., J. Biol. Chem.*, **109**, p. xcvi (1935); **114**, p. cvii (1936). Ackermann, D., *Z. physiol. Chem.*, **239**, 231 (1936). Borsook, H., Dubnoff, J. W., Lilly, J. C., and Marriott, W., *J. Biol. Chem.*, **138**, 405 (1941).
23. Achelis, W., *Z. physiol. Chem.*, **50**, 19 (1907). Kutscher, F., and Lohmann, A., *Z. physiol. Chem.*, **49**, 81 (1906). Engeland, R., *Z. physiol. Chem.*, **57**, 49 (1908). Wada, S., *Acta schol. med. univ. imp. Kioto*, **13**, 187 (1930).
24. Mueller, H., *Z. Biol.*, **83**, 239 (1925).

INTERACTION BETWEEN PROTEINS AND SYNTHETIC DETERGENTS

II. ELECTROPHORETIC ANALYSIS OF SERUM ALBUMIN-SODIUM DODECYL SULFATE MIXTURES*

By FRANK W. PUTNAM AND HANS NEURATH

(*From the Department of Biochemistry, Duke University School of Medicine, Durham, North Carolina*)

(Received for publication, March 21, 1945)

Many biological reactions depend on interaction between proteins or between proteins and other ionizable organic compounds of high or low molecular weight. Characteristic examples of protein-protein interaction are found in certain antigen-antibody reactions, in the combination of enzymes with protein substrates, and in the serum protein system. Complex formation of proteins with protamines or with nucleic acids exemplifies combination with other high molecular weight compounds, while conjugation with acidic or basic prosthetic groups as in the respiratory, lipo-, and glycoproteins represents the reaction with lower molecular weight substances. Moreover, recent work indicates that certain ionizable bacteriostatic agents, such as penicillin (2) and the sulfonamides (3), as well as ionic bactericides, such as the synthetic detergents (1, 4–6), combine with the plasma proteins.

While most cases of complex formation with proteins may be satisfactorily explained on the basis of electrostatic attraction between molecules of opposite sign of charge, other examples exist in which interaction persists in the pH region in which both the protein and the other large organic ion are of like sign. The question arises as to what are the forces and foci of attraction and what significance should be attached to the union.

An explanation may be sought in the concept of the relative affinity of anions for proteins, first proposed by Steinhardt *et al.* (7). Among the anions of highest affinity are long chain sulfates and sulfonates which have been shown to be both excellent protein precipitants (1) and denaturants (6, 8). The purpose of this investigation has been to study the system crystalline horse serum albumin-sodium dodecyl sulfate as a simple analogue of both the general phenomena of protein interaction with ionizable compounds and of protein denaturation. In the first paper of this

* The first paper of this series described the effect of detergents on the precipitation of horse serum albumin (1).

A preliminary account of this work has been given (*Federation Proc.*, 4 (1945), in press).

series (1) it was shown that on the acid side of the isoelectric point the precipitation of serum albumin was dependent on the mole ratio of detergent to protein. Subsequent viscosity studies have indicated that denaturation on the basic side of the isoelectric point was likewise dependent on this ratio (9). These findings have prompted an electrophoretic investigation of the interaction between these two compounds, the results of which are presented herein. The third communication of this series[1] describes an investigation of the molecular kinetic properties of the resulting complexes.

EXPERIMENTAL

Materials and Methods—The materials used in this investigation were five times crystallized horse serum albumin,[2] containing 0.1 per cent carbohydrate, prepared as previously described (1), and purified sodium dodecyl sulfate.[3] Stock solutions were prepared by dissolving weighed amounts in phosphate-NaCl buffer, pH 6.8 (determined with the glass electrode at 25°). The composition of the buffer was 0.025 M NaH_2PO_4, 0.025 M Na_2HPO_4, and 0.10 M NaCl, ionic strength 0.2. The nitrogen content of the protein solutions was ascertained by Kjeldahl analysis. The Tiselius electrophoresis apparatus used in this investigation was equipped with the Philpot-Svensson optical system and has already been described (10).

The serum albumin was electrophoretically homogeneous in the buffer of pH 6.8, both at 1° and 20° (see Experiment 1, Fig. 1, and Experiment 7, Fig. 2). The dodecyl sulfate migrated with a single boundary at 20° (Experiment 8, Fig. 2) but could not be analyzed at 1° because of its limited solubility (about 0.05 per cent). However, in the presence of protein, sodium dodecyl sulfate was soluble at 1° at concentrations as high as 1 per cent, provided the proportion of detergent to protein did not exceed about 0.45 to 0.50 gm. of detergent per gm. of serum albumin. If this proportion was exceeded, even at low absolute detergent concentrations, a crystalline precipitate was obtained, which was identified as alkyl sulfate. These preliminary experiments defined the concentration range suitable for electrophoretic study of complex formation at 1°.

Electrophoresis at 1°—Protein-detergent mixtures of constant detergent concentration (0.30 per cent) and varying protein concentration were prepared by mixing the stock solutions and diluting with buffer to a known

[1] Manuscript in preparation.

[2] We are indebted to the Lederle Laboratories, Inc., Pearl River, New York, for a supply of horse serum.

[3] Obtained through the courtesy of Dr. S. Lenher of the Fine Chemicals Division of E. I. du Pont de Nemours and Company, Inc., Wilmington, Delaware. For the chemical analysis see the preceding paper (1).

volume. To insure complete interaction, the solutions were allowed to stand overnight at room temperature. Dialysis of the mixtures was omitted in order to avert loss of detergent and concomitant changes in composition. This expedient was justified since (*a*) the measured conductivities of the buffer and the solutions differed by less than 1 per cent, and (*b*) the δ- and ε-boundaries did not exceed the usual order of magnitude (*cf.* Fig. 1).

Representative electrophoretic diagrams, printed from the original plates, are given in Fig. 1. The initial boundary is represented by the pair of vertical lines, and is distinguished on the ascending (left) and descending (right) sides, respectively, by the slow moving δ- and ε-boundaries (11). The arrows indicate the direction of migration. Since all of the photographs were taken after electrophoresis for the same length of time (14,400 seconds) and at essentially the same field strength (4.66 to 4.72 volts per cm.), the mobilities of the respective components may be compared directly by inspection of the distance of migration from the origin. The components, labeled *A*, *1*, *2*, and *D*, were identified by their respective mobilities, the relative constancy of which may be ascertained by reference to Table I.

Mobilities were determined from the descending pattern at several different times of electrophoresis and were found to be constant within the experimental error.[1] Mobilities calculated from the ascending pattern likewise showed satisfactory constancy throughout a given experiment, as well as from experiment to experiment.

Inspection of Fig. 1 reveals several boundaries which, in order of increasing mobility, correspond to albumin, labeled *A*, a first complex, *1*, a second complex, *2*, and a trace of material, *D*, believed to be detergent. The relative proportions of the components, as determined by planimeter measurements of the area from tracings of the enlarged photographs, are given in Table I.[5] Since the specific refractive index increments of

[1] Mobilities were calculated in the customary manner from measurements of the displacement from the origin of the maximum ordinate of the constituent boundaries. For diffuse boundaries, not completely separable by electrophoresis, mobilities were determined by measurement of the displacement of curves resolved in the manner of Tiselius and Kabat (12). In this investigation no attempt was made to correct mobilities derived from the ascending pattern for the dilution at the δ-boundary, nor to correct mobilities obtained from the descending pattern for the viscosity of the protein-detergent mixtures.

[5] The center of the illuminated boundary lines was used in the measurements of areas (private communication by Dr. G. R. Cooper). Overlapping gradients were resolved by the method of Tiselius and Kabat (12). Analysis of the area distribution of sharp gradients was facilitated by reducing the slit angle from the conventional 45° used in Fig. 1. We are indebted to Mrs. Jane Sharp for carrying out some of the graphical analyses.

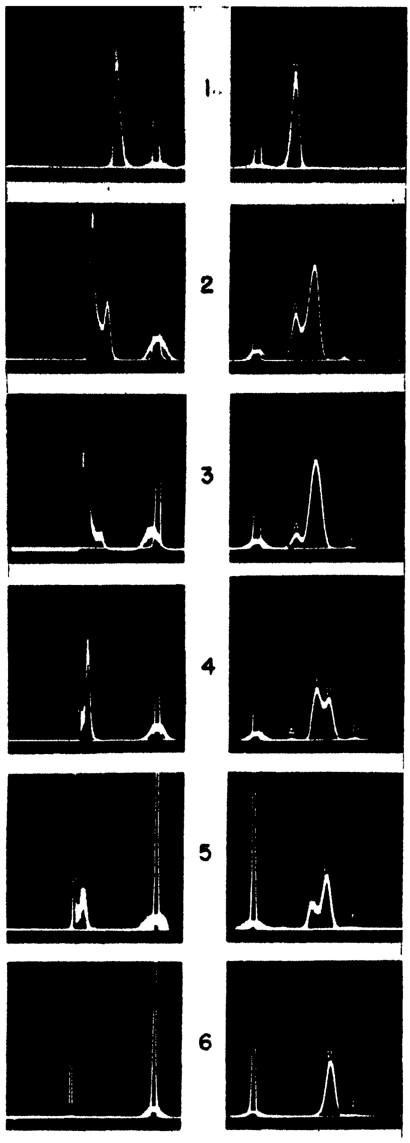


FIG. 1

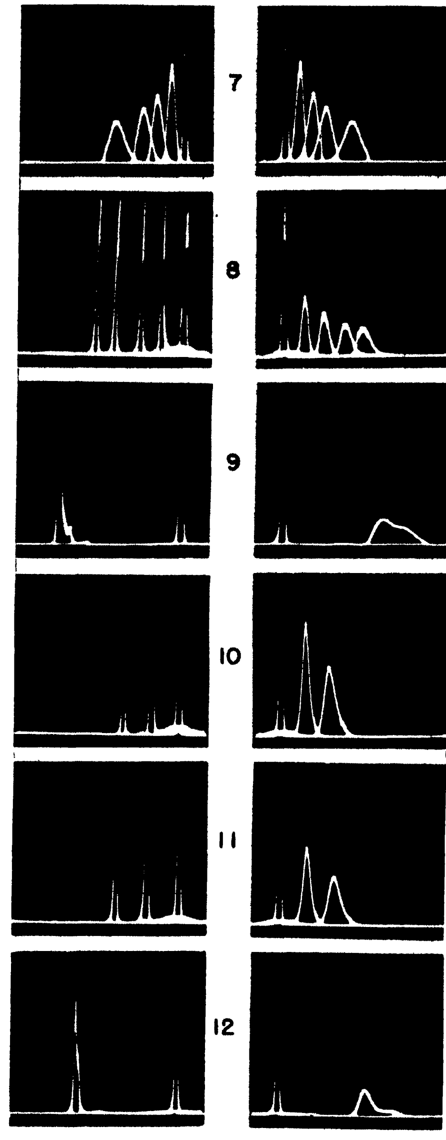


FIG. 2

FIG. 1. Electrophoretic analysis of serum albumin-sodium dodecyl sulfate mixtures at 1°. For the composition of the mixtures in Experiments 1 to 6 see Table I. Since all experiments were run for the same time (14,400 seconds) at essentially the same field strength (4.66 to 4.72 volts per cm.), the constancy of the mobilities of components *A*, *1*, *2*, and *D* may be observed by comparing directly the displacements of the respective boundaries from the common origin (starting boundary, characterized by the pair of vertical lines). The arrows denote the direction of migration; right,

serum albumin and sodium dodecyl sulfate differ (see below), the area distribution only approximates the relative weight proportion of the components.

The proportion of the components, and therefore their composition, does not change with time of electrophoresis. Area distribution measurements on the descending side after 4 hours of electrophoresis give the same results as after 2 hours. Moreover, the distribution among the components is nearly the same on both sides of the cell (Table III). However, small differences are evident; *i.e.*, traces of *A* and *D* on the descending side and boundary splitting of complex *1* on the ascending side. It is noteworthy that the distribution among components is not a function of detergent concentration but merely of detergent to protein ratio (compare Experiments 2 and 2a, Table I).

TABLE I

Electrophoretic Analysis of Serum Albumin-Sodium Dodecyl Sulfate Mixtures at 1°*

Experiment No.	Composition		Relative area†				Mobility, 10^{-5} sq. cm. volt^{-1} sec.$^{-1}$			
	Albumin	Detergent	*A*	*1*	*2*	*D*	*A*	*1*	*2*	*D*
	gm. per cent	*gm. per cent*	*per cent*	*per cent*	*per cent*	*per cent*				
1	0.81	0	100				4.9			
2	1.98	0.30	26.0	72.5		(1.4)	5.1	7.5		(11.8)
2a‡	0.99	0.15	26.4	73.6			5.2	7.8		
3	1.49	0.30	10.0	89.6		(0.4)	5.3	7.8		(12.5)
4	0.99	0.30	(0.6)	57.5	41.0	(0.9)	(4.9)	8.1	9.8	(13.1)
5	0.79	0.30	(0.7)	28.0	69.6	(1.6)		8.0	9.9	(13.0)
6	0.59	0.30			98.0	(2.0)			10.4	(13.4)

The values given in parentheses are only approximate, since they approach the limit of optical resolving power of the apparatus.

* All experiments were performed in phosphate-NaCl buffer, $\mu = 0.2$, pH 6.8, at 1° for 4 hours at constant field strength varying from experiment to experiment between 4.66 and 4.72 volts per cm. Data calculated from descending pattern after 2 hours migration.

† These figures refer to area proportions only and not to weight percentage composition (see the text).

‡ 4 hours migration; *D* boundary migrated out of the visual field.

Electrophoresis at 20°—Analysis of the electrophoretic data obtained at 1° was hindered by the lack of a mobility value for pure sodium dodecyl

descending; left, ascending boundary. The figures between the patterns represent the experiment numbers.

FIG. 2. Electrophoretic analysis of serum albumin-sodium dodecyl sulfate mixtures at 20°. For the composition of mixtures in Experiments 7 to 12 see Table II. Exposures made at different times of migration are superimposed on the same plate. The arrows denote the direction of migration; right, descending; left, ascending boundary.

sulfate and of a factor relating area to weight concentration. It was also uncertain whether complexes *1* and *2* had the same composition at room temperature, where viscosity and diffusion measurements were made,[1] as at 1°, and whether in higher concentrations than could be obtained at 1° additional detergent was bound to complex *2*. Accordingly, electrophoretic measurements were undertaken at 20°.

Tiselius' early experiments show that at 20° heat convection may be minimized by reduction in potential gradient and increase in time of electrophoresis and that the maximum allowable heat effect is about 10×10^{-3} watt per cc. (13). As a test for the absence of convection currents, pure serum albumin was exhaustively dialyzed against buffer (pH 6.8) and subjected to electrophoresis at 20° for 20 hours at 0.428 volt per cm. Calculations show that under these conditions the resulting heat effect is below the critical level and but slightly higher than that obtained by Tiselius in his work at 20° with the thermally relatively inefficient cylindrical cell.

Photographs were taken successively after 4, 8, 12, and 20 hours, and superimposed on the same plate (Experiment 7, Fig. 2). These patterns are distinguished by a notable symmetry of the ascending and descending boundaries and by the absence of any visible δ- and ϵ-boundaries. The mobilities on each side and the areas under the individual boundaries remained constant with time. The mobility values calculated from the ascending and descending patterns were in excellent agreement with each other; *i.e.*, 10.02 and 9.86×10^{-5} sq. cm. volt^{-1} sec.$^{-1}$, respectively (uncorrected for viscosity). The slight skewness of the boundaries is also observable at 1° on prolonged electrophoresis and is probably related to the recognized electrophoretic complexity of crystalline horse serum albumin (14). It follows, therefore, that under the conditions just described, thermal convection played no significant part in the electrophoretic experiments at 20°.

The electrophoretic pattern of a 1.20 per cent solution of undialyzed sodium dodecyl sulfate in phosphate buffer of pH 6.8 is shown in Fig. 2, Experiment 8. Here, again, the mobilities remained constant with time though the ascending boundary migrated faster than the descending one. The tendency of the former to become sharper, and of the latter to become more diffuse, may be attributed to the enhanced dilution effect at the δ-boundary as well as to an electrophoretic disturbance of the micelle-molecule equilibrium of the detergent.

In order to determine whether the composition of complexes *1* and *2* was the same at 20° as at 1°, a mixture of albumin and detergent corresponding to that used in Experiment 4 (Table I) was studied at 20°. Area measurements yielded values of 56 per cent for complex *1* and 44 per cent for complex *2*, in good agreement with those obtained at 1°. The com-

ponents were identified by their mobilities (corrected to 1°).[6] Since, at the same mixing ratio, the component distribution was approximately the same at the two temperatures, their composition must be essentially independent of temperature.

Since at 20° sodium dodecyl sulfate-albumin mixtures were found to be soluble at pH 6.8 in all proportions, mixtures were prepared in which the detergent to albumin ratio exceeded that corresponding to complex *2*, with the object of determining the presence and composition of higher complexes. In Experiments 10 to 12, the albumin concentration was varied over a 3-fold range, at a constant detergent concentration of 0.6 per cent. These concentrations correspond approximately to detergent-protein ratios of, respectively, $\frac{2}{3}$, 1, and 2, as compared to the ratio of $\frac{1}{2}$ for the homogeneous

TABLE II

Electrophoretic Analysis of Serum Albumin-Sodium Dodecyl Sulfate Mixtures at 20°*

Experiment No.	Composition		Time	Mobility,† 10^{-5} sq. cm. volt^{-1} sec.$^{-1}$				
	Albumin	Detergent		*A*	*1*	*2*	AD_x	*D*
	gm. per cent	*gm. per cent*	*hrs.*					
7	0.72	0	4, 8, 12, 20	5.8				
8	0	1.20	2, 4, 6, 8					18.1
9	0.99	0.30	22		8.0	9.4		
10	0.84	0.60	4, 8				10.6‡	
11	0.56	0.60	4, 8				11.8‡	
12	0.28	0.60	12				13.0‡	17.0

* All experiments were performed in phosphate-NaCl buffer, $\mu = 0.2$, pH 6.8, at 20°, at constant field strength varying from experiment to experiment between 0.403 and 0.434 volt per cm. Data for descending boundary only.

† Mobilities corrected to 1° (see the text).

‡ Calculated from the displacement of the maximum ordinate.

complex *2* at 1° (Experiment 6). While Experiments 10 and 11 yielded single boundaries, close inspection of the forward edge of the descending pattern reveals a skewness which approaches a definite separation in Experiment 12. Moreover, the calculated mobility of the complex (Table II) increases linearly with the proportion of detergent in the mixture. The mobility of the faster migrating boundary in Experiment 12 approaches that of pure detergent.

[6] In order to facilitate comparison of the experiments at the two different temperatures, mobilities at 20° were converted to 1° by a factor (0.59), account being taken of the viscosity difference of the solvent (water). This factor was approximately equal to the ratio of the conductivities of the buffer at the two temperatures (0.61). This correction is approximate, because it does not take into account the temperature coefficients of the dissociation constants of the protein and the buffer or the effect on the micelle-molecule equilibrium of the detergent.

In Experiment 12, electrophoresis was definitely anomalous in regard to asymmetry of patterns on the two sides of the cell. While a precise value could not be assigned to the mobility on the ascending side, the approximate value is intermediate between that exhibited by the slower moving complex and the faster moving detergent on the ascending side.

Composition of Complexes—The dependence of the precipitability, viscosity, and electrophoresis of protein-detergent mixtures on the weight ratio of the two substances, rather than on their absolute concentrations, suggests the formation of stoichiometric complexes of discrete composition. To test this hypothesis, an attempt was made to calculate the composition of the electrophoretic complexes by the method of Longsworth and MacInnes (15). It was realized, however, that the presence of four possible components in this system rendered such calculations hazardous.

Component composition was calculated in the following manner. Arbitrary factors relating weight concentration to area units were obtained for pure serum albumin and sodium dodecyl sulfate at 20° by planimetric integration of the tracings of the enlarged electrophoretic diagrams. These factors depend on the specific refractive index increments of the substances in solution and on apparatus and planimeter constants. The factors obtained were, for albumin, $K_A^{20} = 5.56 \times 10^{-3}$, and for detergent, $K_D^{20} = 10.65 \times 10^{-3}$. The products of these arbitrary constants and of the respective specific refractive index increments measured in the phosphate buffer with an Abbe refractometer were in good agreement; *i.e.*, 1.16 and 1.23×10^{-6}.

In the same manner, a constant was calculated for pure serum albumin at 1°, $K_A^1 = 5.6_4 \times 10^{-3}$. With this factor, the concentration of free albumin in mixtures of albumin and complex *1* was calculated from its area after correction for the δ dilution effect. The result is given on Line 8 of Table III. This amount was subtracted from the total albumin concentration, yielding the amount of albumin combined (Line 9, Table III). It was assumed that all detergent present was combined, the trace of unknown component D being neglected. The ratio of these two values gives the composition of complex *1* in gm. of detergent per gm. of albumin (Line 10, Table III). The values obtained for Experiments 2 and 3 are, respectively, 0.21 and 0.22. It is of interest that, if the difference in refractive index increments of the components be neglected, the composition of complex *1* in the two experiments is calculated to be 0.22 and 0.23 gm. of detergent per gm. of albumin. Accordingly, 0.22 gm. of detergent per gm. of albumin was accepted as a fair estimate of the composition of complex *1*.

An estimate of the composition of complex *2* was made in the same manner. However, it was first necessary to calculate the arbitrary area

constant of complex *1* from the areas and concentrations given in Table III ($K_1^1 = 7.2 \times 10^{-3}$). Now, with the assumption that in mixtures of *1* and *2* the composition of complex *1* remained constant, the composition of complex *2* was calculated by difference. For Experiments 4 and 5, the values of 0.44 and 0.45 gm. of detergent per gm. of albumin were obtained. In order to calculate a value from Experiment 6, it had to be assumed that the refractive index increment of pure detergent was the same at 1° as at 20°, and that component *D* represented pure detergent. The resulting value was 0.45 gm. of detergent per gm. of albumin.

TABLE III

Representative Calculation of Composition of Serum Albumin-Sodium Dodecyl Sulfate Complex from Electrophoretic Measurements at 1°

Line No.		Experiment 1		Experiment 2		Experiment 3	
		Ascending	Descending	Ascending	Descending	Descending, 2 hrs.	Descending, 4 hrs.
		area units	*area units*	*area units*	*area units*	*area units*	*area units*
1	*A**	133.2	138.9	71.3	78.2	22.4	20.1
2	*1*			185.6	218.5	201.9	202.1
3	*2*						
4	*D*				(4.2)	(1.0)	(1.4)
5	δ or ϵ	11.4	5.6	63.4	26.4	21.6	19.1
6	Total	144.6	144.5	320.3	327.3	246.9	242.7
		gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
7	*A* taken	0.81	0.81	1.98	1.98	1.49	1.49
8	" free	0.81	0.81	0.56	0.48	0.14	0.13
9	" combined	0	0	1.42	1.50	1.35	1.36
10	*D* per gm. *A* in complex *1*			0.21	0.20	0.22	0.22

The values given in parentheses are only approximate, since they approach the limit of optical resolving power of the apparatus.

* *A*, *1*, *2*, and *D* denote, respectively, albumin, complexes *1* and *2*, and detergent.

A test for the constancy of composition of the complexes was based on the assumption that the percentage distribution of the areas closely approximated the percentage distribution of the concentrations of the individual components. The error involved in this assumption was small, since (*a*) mixtures of detergent and albumin gave patterns in which either the pair albumin-complex *1* or complex *1*-complex *2* was present, and (*b*) for each pair the measured refractive index increments of the components differed by but 10 per cent. At mixing ratios intermediate between those corresponding to either pure albumin and pure complex *1*, or pure complex

1 and pure complex *2*, the area distribution between the components was found to be a linear function of the mixing ratios (data of Table I).

Calculations of the composition of the complexes formed at 20° in solutions with detergent to albumin ratios of $\frac{2}{3}$, 1, and 2 were hindered by increasing skewness of the descending boundary. Since only at the highest ratio studied was free detergent observable, it may be assumed that above a mixing ratio corresponding to complex *2* complexes of variable composition are formed up to a mixing ratio of about 1. This interpretation is supported by the data of Experiment 12 from which the combining ratio of the complex, represented by the slower moving boundary, was calculated to be 0.28 gm. of detergent per 0.28 gm. of albumin. The skewness of the boundaries observed in these experiments may be ascribed to partial dissociation of loosely bound detergent under the influence of the electric field.

DISCUSSION

Studies of the interaction of proteins and other colloids have revealed the phenomenon to be predominant in the pH region in which the particles bear opposite net electrical charges. For mixtures of proteins and other amphoteric substances (first type) interaction is generally restricted to the interisoelectric region, whereas, for mixtures of proteins and *high* molecular weight anions (second type), complex formation usually occurs only on the acid side of the isoelectric point or in the isoelectric range. (The analogous case of the interaction between high molecular weight cations and proteins is also to be recognized.) It is reasonable to assume that where interaction is restricted to the pH region of opposite net charge, the operating forces are electrostatic (interionic), whereas, if interaction extends to pH regions at which the reacting components carry net charges of like sign, the operation of forces not wholly ionic may be admitted.

However, as has been pointed out in a discussion of this problem (16), a third type of ionic interaction may exist; *i.e.*, "if one component of a mixture were protein (amphoteric) while the other were actually or potentially a relatively low molecular weight electrolyte (such as a detergent), ionic interaction could occur in ranges of pH where both components possessed the same sign of net charge provided there still existed ionized groups, on the protein, having a sign of charge opposite to that of the net charge."

Several systems exemplifying each of these three types of interaction have been studied electrophoretically; *e.g.*, the formation of protein-protein complexes in egg white in the interisoelectric region (first type (17)), complex formation between egg albumin and yeast nucleic acid (15) which ceases just above the isoelectric point (second type), and between

egg albumin and detergent mixtures on the alkaline side of the isoelectric point (third type (5)).

It should be observed that the formation of stoichiometric complexes, the composition of which is independent of pH and absolute concentration, proceeds most readily in the third type of interaction. For, it is the only case in which combination may be postulated to occur between the ion and individual charged groups on the protein. Though the net charge of the amphoteric protein varies with pH, there is a wide region in which all groups capable of salt-like combination with a low molecular weight ion are fully ionized. The extent of combination, as studied by electrophoresis, depends (*a*) on the affinity of the anion for the protein, and (*b*) on the degree to which the union resists dissociation under the influence of the electric field. Longsworth and MacInnes (15) have discussed the manner in which the equilibrium will shift to meet the altered conditions produced by electrophoretic separation.

The electrophoretic behavior of mixtures of serum albumin and sodium dodecyl sulfate is characterized by three phenomena: (*a*) at pH 6.8 not one but two complexes of definite composition are formed, their distribution depending on detergent to protein ratio; (*b*) at 1°, practically all of the detergent is combined; (*c*) the mobility of the components and the per cent area distribution are constant with time, and are comparable for the two sides of the cell. The symmetry of the ascending and descending patterns, with regard to number, displacement, and area distribution of boundaries is an indication of the formation of well defined complexes.

Since an equilibrium exists between detergent in the micellar state (molecular weight about 20,000)[1] and in the unaggregated form (molecular weight 288.4), binding of single detergent anions with individual reactive protein groups may take place stoichiometrically. The composition of complex *1* as determined by electrophoresis at pH 6.8 (*i.e.*, 0.22 gm. of detergent per gm. of albumin) corresponds to the minimum ratio required for complete precipitation of the protein at pH 4.5 (1). Similarly, the electrophoretic composition of complex *2* at pH 6.8 (*i.e.*, 0.45 gm. of detergent per gm. of albumin) closely approximates the maximum ratio producing complete precipitation at pH 4.5. Intermediate mixing ratios correspond to the "equivalence zone" of the precipitation curve, and to varying distribution of the two complexes observed in electrophoresis. In the study of the viscosity behavior of protein-detergent mixtures, a similar correlation has been noted.[1] This correspondence of data suggests that combination is independent of pH within the range 4.5 to 6.8. Accordingly, interaction must involve protein groups, presumably cationic, which remain fully ionized in this region.

Recent analytical data of Brand *et al.* (18) indicate that horse serum

albumin (carbohydrate-free Fraction B) contains 117 basic groups per mole (molecular weight 70,000). Of these, 62 or about one-half are strongly basic (guanidinium and ϵ-amino) and are positively charged at reactions acid to pH 8 (19); of the remainder, thirty-five (α-amino) are practically fully ionized at pH 6.8, and the rest (twenty imidazole) are about 50 per cent ionized. Accordingly, about 110 groups are positively charged at this pH. If the composition of complex *2* is taken as 0.45 gm. of detergent per gm. of albumin, it may be calculated that this corresponds to the binding of 109 moles of sodium dodecyl sulfate per mole of albumin; *i.e.*, each positively charged group binds 1 detergent anion. Similarly, the composition of complex *1* (0.22 gm. of detergent per gm. of albumin) corresponds to the binding of 54 detergent anions; *i.e.*, equivalent to one-half the number of positively charged groups.[7]

Steinhardt *et al.* (7) have already shown that the anions of many strong acids undergo a reversible stoichiometric combination with both soluble and insoluble proteins. Thus, dodecyl sulfonate and dodecyl sulfate are almost quantitatively transferred from the solution to the protein in an amount equivalent to the sum of the basic groups of the proteins. The relationship of stoichiometric anion combination to the precipitation of proteins is also borne out by the observation that, in metaphosphoric acid protein precipitates, the combined metaphosphate is equivalent to the number of positively charged groups of the protein (22).

In a subsequent paper[1] the structure of the stoichiometric complexes of serum albumin and sodium dodecyl sulfate will be discussed. Suffice it to point out here that the protein groups which combine to form complex *1* may differ from the remainder in basicity, accessibility, or structural function. It is recognized that the experimental error involved in the measurement of electrophoretic component distribution places limits upon an accurate determination of the composition of these complexes, and that the subsequent designation of complexes *1* and *2* as, respectively, AD_n and AD_{2n}, or AD_{55} and AD_{110}, is a convenient approximation.

The close correspondence of the number of positively charged protein groups with the number of detergent anions bound per mole is good indication that ionic forces are involved in interaction. It should be pointed out that the apparent additivity of the net charges of detergent and protein,

[7] It is of interest to calculate the number of moles of Nacconol bound per mole of egg albumin (5). If the complex is assumed to have a composition of 25 per cent detergent of an average molecular weight of 323, and egg albumin a molecular weight of 45,000, the calculated mole ratio is 46, or approximately 1 mole of detergent for each of the forty-one cationic groups of egg albumin (20). In this connection it is also noteworthy that surface film measurements (21) indicate the formation of two well defined complexes of sodium dodecyl sulfate with egg albumin, one containing 17 moles of detergent per mole, the other one 32 moles.

as evidenced by the intermediate mobilities of the complexes, is no proof that the ionizable groups of each component are free and that non-polar forces are responsible for attraction (23). With the binding of each detergent anion, the resultant contribution to the net charge is the same whether the anion is bound by non-polar forces and the charged group remains free, or whether it reacts with a single positively charged protein group, thereby masking the latter's charge.

The present data fail to reveal the exact composition of complexes higher than AD_{110}, or the forces involved in their formation. Although at 20° single boundaries have been obtained when the detergent to albumin ratio was varied within the range of 0.45 (corresponding to complex AD_{110}) to 1.0, it appears that in this range some detergent anions are loosely bound. This is evidenced (*a*) by the skewness of the boundaries arising upon prolonged electrophoresis, and (*b*) by the ease with which detergent may be removed by shifting the equilibrium between free and combined detergent by freezing. The evidence for the latter is as follows:

Two detergent-protein mixtures, corresponding in composition to, respectively, AD_{2n} (0.84 per cent detergent and 1.86 per cent albumin) and AD_{4n} (1.67 per cent detergent and 1.86 per cent albumin), were prepared at room temperature and, after 24 hours standing, were stored at 0° for 4 days. No precipitation was evident in the former, while a copious precipitate formed in the latter. The precipitate, identified as sodium dodecyl sulfate, was removed by centrifugation in the cold, and the supernatant solution analyzed electrophoretically at 1° in 2-fold dilution. The electrophoretic pattern revealed the presence of a major boundary, corresponding in mobility not to AD_{2n} but to AD_n, a minor component, similarly identified as albumin, and a trace of AD_{2n}. The fact that detergent can be removed by freezing from complexes higher than AD_{2n} indicates that the binding is of a looser type. However, since the resulting component is predominantly AD_n rather than AD_{2n}, it follows that the phase transition of free detergent to the solid form has forced the dissociation of the complexes. Essentially the same result can be achieved by partial removal of detergent by prolonged dialysis. Moreover, complete regeneration of albumin can be accomplished by precipitation of the detergent as its barium salt (1). These results indicate that although sodium dodecyl sulfate has a high affinity for proteins, the complexes are dissociable.

With regard to the nature of the forces involved in the formation of complexes higher than AD_{2n}, it is of interest to note that it is in this range of detergent to albumin ratios that the precipitate formed in acid solutions becomes soluble. Moreover, in this range a large increase in intrinsic viscosity of the protein is observed which may be attributed to profound disorientation of the protein molecule. These aspects of the problem will be discussed in a subsequent paper.[1]

Preliminary experiments with mixtures of serum albumin and pure lower homologous alkyl sulfates (*i.e.*, octyl and decyl) are in accord with Steinhardt's conclusions with regard to the dependence of anion affinity on chain length (7). Thus while both octyl and decyl sulfate were capable of causing complete precipitation of the protein at pH 4.5, the effective concentration, compared on an equimolar basis, was several times higher than that of the dodecyl homologue, uncombined detergent remaining in solution. Anomalies resulting from the increased dissociation tendency of the resulting complexes during electrophoresis precluded quantitative determination of component distribution. It is therefore apparent that mixtures of homologous detergents do not lend themselves to a study of this type.

This work was supported by grants from the Rockefeller Foundation, the Lederle Laboratories, Inc., and the Duke University Research Council. We are indebted to the Division of Experimental Surgery of Duke University School of Medicine for making available to us the Tiselius apparatus used in this investigation.

SUMMARY

As a simple analogue to the interaction between proteins and other ionized biological compounds, the system crystalline horse serum albumin-sodium dodecyl sulfate has been studied electrophoretically. In a phosphate-NaCl buffer, pH 6.8, $\mu = 0.2$, two discrete complexes have been identified at 1°, while at 20° additional complexes of varying composition have been observed. The distribution among the complexes depends only on the detergent to protein weight ratio. The number of detergent anions bound in the formation of the first two complexes has been found to be equivalent to, respectively, one-half and all the cationic protein groups. This stoichiometric combination of detergent anions with basic protein groups explains the specific precipitation of proteins by anions of high affinity. The forces involved in the formation of the first two complexes are believed to be ionic. The general phenomenon of protein interaction has been discussed in the light of the present findings.

The higher complexes of variable composition are electrophoretically monodisperse up to a detergent to protein ratio of 1, at which free detergent first makes its appearance. The nature of these relatively easily dissociable complexes has been considered in relation to the precipitation and viscosity behavior of the protein.

BIBLIOGRAPHY

1. Putnam, F. W., and Neurath, H., *J. Am. Chem. Soc.*, **66**, 692 (1944).
2. Chow, B. F., and McKee, C. M., *Science*, **101**, 67 (1945).

3. Davis, B. D., *J. Clin. Invest.*, **22**, 753 (1943).
4. Kuhn, R., Bielig, H. J., and Dann, O., *Ber. chem. Ges.*, **73 B**, 1080 (1940).
5. Lundgren, H. P., Elam, D. W., and O'Connell, R. A., *J. Biol. Chem.*, **149**, 183 (1943).
6. Putnam, F. W., and Neurath, H., *J. Biol. Chem.*, **150**, 263 (1943).
7. Steinhardt, J., Fugitt, D. H., and Harris, M., *J. Res. Nat. Bur. Standards*, **26**, 293 (1941); **28**, 201 (1942).
8. Anson, M. L., *J. Gen. Physiol.*, **23**, 239 (1939).
9. Putnam, F. W., and Neurath, H., *J. Am. Chem. Soc.*, **66**, 1992 (1944).
10. Sharp, D. G., Cooper, G. R., and Neurath, H., *J. Biol. Chem.*, **142**, 203 (1942).
11. Longsworth, L. G., and MacInnes, D. A., *J. Am. Chem. Soc.*, **62**, 705 (1940).
12. Tiselius, A., and Kabat, E. A., *J. Exp. Med.*, **69**, 119 (1939).
13. Tiselius, A., *Nova acta reg. soc. sc. Upsaliensis*, **7**, No. 4 (1930).
14. Sharp, D. G., Cooper, G. R., Erickson, J. O., and Neurath, H., *J. Biol. Chem.*, **144**, 139 (1942).
15. Longsworth, L. G., and MacInnes, D. A., *J. Gen. Physiol.*, **25**, 507 (1942).
16. Schwert, G. W., Putnam, F. W., and Briggs, D. R., *Arch. Biochem.*, **4**, 371 (1944).
17. Longsworth, L. G., Cannan, R. K., and MacInnes, D. A., *J. Am. Chem. Soc.*, **62**, 2580 (1940).
18. Brand, E., Kassell, B., and Saidel, L. J., *J. Clin. Invest.*, **23**, 437 (1944).
19. Cohn, E. J., and Edsall, J. T., Proteins, amino acids and peptides, American Chemical Society monograph series, New York, 445 (1943).
20. Cannan, R. K., Kibrick, A., and Palmer, A. H., *Ann. New York Acad. Sc.*, **41**, 243 (1941).
21. Bull, H. B., *J. Am. Chem. Soc.*, **67**, 10 (1945).
22. Perlmann, G. E., *J. Biol. Chem.*, **137**, 707 (1941).
23. Palmer, K. J., *J. Phys. Chem.*, **48**, 12 (1944).

THE SEPARATION OF PURINE NUCLEOSIDES FROM FREE PURINES AND THE DETERMINATION OF THE PURINES AND RIBOSE IN THESE FRACTIONS

BY STANLEY E. KERR AND KRIKOR SERAIDARIAN

(From the Department of Biological Chemistry, American University of Beirut, Beirut, Lebanon)

(Received for publication, March 15, 1945)

A method was previously described for the estimation of total nucleoside and free purine nitrogen in the trichloroacetic acid extracts of blood and tissues, after removal of nucleotides by precipitation with uranyl acetate (1, 2). This procedure gives no indication as to the amount of purine present as nucleoside. The further separation of nucleoside from free purine and the analysis of each of these fractions for individual purines and for ribose are now described in this paper.

EXPERIMENTAL

The free purine bases may be separated from the purine nucleosides by precipitation with silver nitrate in dilute acid solution. When the solution is treated with sufficient excess of NaOH to precipitate a little silver oxide (final concentration about 0.002 N), the nucleosides are quantitatively precipitated. Nucleotides and free purines, if not previously removed, are likewise precipitated by $AgNO_3$ in alkaline solution.

Since this procedure was designed for the analysis of trichloroacetic acid extracts of tissues, all of the studies on precipitation by silver nitrate have been carried out in the presence of 8 per cent trichloroacetic acid, first neutralized and then adjusted to the desired acidity. In each experiment 5 cc. of an analyzed solution of the compound to be tested were added to 20 cc. of neutralized 10 per cent trichloroacetic acid, the acidity was adjusted, and 0.5 cc. of molar $AgNO_3$ solution was added. The precipitate was separated by centrifugation, washed once, then ashed, and the nitrogen content determined by a micro-Kjeldahl procedure (2).

The effect of varying acidity on the precipitation by $AgNO_3$ is reported in Table I. In solutions made slightly alkaline with NaOH each of the purines and nucleosides tested and myoadenylic acid were completely precipitated. In neutral solution myoadenylic acid and guanosine were precipitated completely, adenosine only partially. Acidity as low as 0.016 N acetic acid (0.1 per cent) entirely prevented the precipitation of adenosine and inosine, but permitted the precipitation of some myoadenylic acid. Inosine also precipitated on standing overnight, but not

within an hour. At least 95 per cent of the purine bases, adenine, hypoxanthine, and guanine, was precipitated at acidities as high as 0.05 N H_2SO_4.

TABLE I

Precipitation of Purines, Nucleosides, and Myoadenylic Acid by Silver Nitrate (0.02 M) in Presence of 8 Per Cent Trichloroacetic Acid at Various Acidities and in Alkaline Solution

Purine	Acidity	pH	Time for pptn.	Purine N taken	N found	Recovery
				mg.	*mg.*	*per cent*
Adenine	0.002 N NaOH		45 min.	0.424	0.422	99.2
	0.016 " acetic	4.1	30 "	0.290	0.285	98.3
	0.02 " H_2SO_4	2.0	30 "	0.415	0.415	100.0
	0.05 " "	1.5	30 "	0.415	0.397	95.5
	0.07 " "	1.4	30 "	0.515	0.476	92.6
	0.10 " "	1.2	30 "	0.515	0.472	91.6
Hypoxanthine	0.002 " NaOH		15 "	0.518	0.511	98.8
	0.016 " acetic	4.1	30 "	0.560	0.543	97.0
	0.04 " H_2SO_4	1.7	30 "	0.526	0.510	97.0
	0.07 " "	1.4	30 "	0.526	0.500	95.2
	0.10 " "	1.2	30 "	0.526	0.472	89.8
Guanine	0.04 " "	1.7	30 "	0.432	0.420	97.3
	0.07 " "	1.4	30 "	0.432	0.419	97.0
	0.10 " "	1.2	30 "	0.432	0.416	96.2
Adenosine	0.002 " NaOH		12 "	0.392	0.387	98.6
	Neutral		60 "	0.395	0.261	66.0
	0.001 N acetic		180 " at 0°	0.395	0.008	2.1
	0.016 " "	4.1	30 "	0.395	None	0
	0.02 " H_2SO_4	2.0		1.914	"	0
Inosine	0.002 " NaOH		30 "	0.218	0.214	98.2
	0.016 " acetic	4.1	60 "	0.218	None	0
	0.016 " "		18 hrs.	0.218	0.153	70.2
Guanosine	0.002 " NaOH		30 min.	0.445	0.437	98.2
	Neutral		30 "	0.445	0.454	102.0
	0.016 N acetic	4.1	30 "	0.452	None	0
	0.02 " H_2SO_4	2.0	30 "	0.452	"	0
Myoadenylic acid	0.002 " NaOH		60 "	0.332	0.327	98.4
	Neutral		60 "	0.332	0.319	96.1
	0.016 N acetic	4.1	18 hrs.	0.332	0.031	9.3
	0.02 " H_2SO_4	2.0	18 "	0.255	None*	

* Silver nitrate produces a slight cloud, but no precipitate.

Although the nucleosides are not precipitated by $AgNO_3$ when treated *alone* in the presence of 0.016 N acetic acid (pH about 4), some precipitation occurs at this acidity when they are mixed with free purines. A better separation is accomplished by increasing the acidity to 0.02 N with sulfuric

acid. Mixtures of purines with nucleosides were treated with $AgNO_3$ at this acidity (pH about 2.0), 8 per cent trichloroacetic acid (neutralized) also being present. After the acid silver precipitate had been twice centrifuged and washed, sufficient N NaOH was added to render the solution slightly alkaline and to form some silver oxide, in order to precipitate the nucleosides, after which the two precipitates were examined for their content of nitrogen. The results are presented in Table II. The nitrogen found in the acid silver precipitate was assigned to free purine, and that in the alkaline precipitate to nucleoside, on the basis of the results presented in Table I. The results indicate a satisfactory separation of purine from nucleoside except in the mixture of guanosine with adenine, in which about 13 per cent of the guanosine was precipitated in the acid solution with

TABLE II

Analyses of Mixtures of Purines and Nucleosides by Precipitation with Silver Nitrate (0.02 M) in Presence of 0.02 N H_2SO_4, and by NaOH in Supernatant Fluid, with Nitrogen Determinations on Two Precipitates

Compounds mixed	Purine N taken	N in acid silver ppt.	N in alkaline silver ppt.	Recovery
	mg.	*mg.*	*mg.*	*per cent*
Hypoxanthine	0.540	0.545		101.0
Adenosine	0.405		0.386	95.6
Hypoxanthine	0.528	0.518		98.3
Adenosine	0.382		0.392	102.5
Hypoxanthine	0.521	0.496		95.5
Adenosine	0.147		0.147	100.0
Adenine	0.416	0.405		97.3
Adenosine	0.385		0.393	102.0
Adenine	0.515	0.506*		98.3
Adenosine	0.400		0.394*	98.7
Adenine	0.416	0.932		100.0
Hypoxanthine	0.516			
Adenosine	0.385		0.400	103.7
Hypoxanthine	0.521	0.511		98.3
Inosine	0.393		0.405	103.0
Adenine	0.515	0.580		112.6
Guanosine	0.544		0.465	85.5
Adenine	0.392	0.388†		99.0
Guanosine	0.452		0.469†	104.0
Guanine	0.432	0.423		97.8
Adenosine	0.400		0.398	99.5
Guanine	0.647	0.648		100.1
Adenosine	0.437		0.427	97.5

* Both precipitates extracted with *cold* 0.5 N HCl seven times.

† Separation made by precipitation in presence of 0.05 N instead of 0.02 N H_2SO_4.

adenine. By increasing the concentration of H_2SO_4 to 0.05 N, a good separation was accomplished.

To make certain that the findings of Table II were not simply fortuitous, the ribose content of the two silver precipitates, as well as that of the nucleoside added to the solution, was determined in a separate series of experiments. The silver precipitates were washed twice, then extracted with hot 0.5 N HCl, and the ribose in the extracts determined colorimetrically as described below. The results (Table III) support the conclusions based

TABLE III

Analyses of Mixtures of Purines and Nucleosides by Precipitation with Silver Nitrate (0.02 M) in Presence of 0.02 N H_2SO_4, and by NaOH in Supernatant, with Determinations of Ribose in HCl Extracts of Two Precipitates

Compounds mixed	Ribose taken	Ribose found in acid Ag ppt.	Ribose found in alkaline Ag ppt.	Recovery
	mg.	*mg.*	*mg.*	*per cent*
Adenosine	0.880		0.817	93.0
Hypoxanthine	0	0.020		2.3
Adenosine	0.880		0.833	94.5
Hypoxanthine	0	0.014		1.6
Adenosine	0.337		0.327	97.0
Hypoxanthine	0	0.010		3.0
Guanosine	1.165		0.893	76.6
Adenine	0	0.173		14.9
Guanosine	0.970		0.917	94.5*
Adenine	0	0.085		8.7*
Adenosine	0.900		0.853	94.7
Guanine	0	None		0

* Separation made by precipitation in presence of 0.05 N instead of 0.02 N H_2SO_4.

on the nitrogen content of the silver precipitates that the free purines, but not the nucleosides, are precipitated by the acid silver reagent, and that the nucleosides are completely precipitated when the filtrate is made alkaline.

Procedure for Analysis of Tissue Extracts—The amount of tissue to be taken for analysis depends on the expected content of nucleoside and free purine. It is desirable to have samples containing about 3 mg. of purine and nucleoside nitrogen, but this is not feasible with fresh tissues. In brain, for example, before autolysis the entire nucleoside and free purine nitrogen amounts to only 2 mg. per 100 gm. (3), though it rises to 10 mg. within an hour. In our studies on tissues we have used samples of 40 to 50 gm. of brain (the entire brain of a dog), and about 35 gm. of other tissues.

The preparation of the trichloroacetic acid extracts of fresh and autolyzed brain (3, 4) and fresh muscle (1) has already been described. In

experiments on muscle, if rapid fixation is not essential, the tissue may be ground in a Latapie mincer, or in a meat chopper and then with quartz sand in a mortar. The minced tissue is then transferred to a volume of 10 per cent trichloroacetic acid sufficient to make a 1 to 10 dilution. After an hour on ice with frequent mixing it is filtered and neutralized to phenolphthalein with NaOH. A record must be kept of the volumes of filtrates used and reagents added at various steps of the procedure to permit final calculation of the percentage content of the individual fractions in the original tissue.

Removal of Nucleotides—As a precipitant for nucleotides, mercuric acetate is unsuitable for use in the present study. From extracts of autolyzed brain, for example, most of the nucleoside and free purine is precipitated by mercuric acetate together with the nucleotide, whereas with muscle extracts only nucleotide is precipitated (3). We were obliged therefore to return to uranyl acetate as the nucleotide precipitant, since it precipitates neither nucleosides nor free purines (5, 1).

The neutralized, protein-free filtrate is made slightly acid with 10 per cent acetic acid (5 drops per 100 cc. of filtrate), and treated with the *minimum* amount of 8 per cent uranyl acetate to provide a slight excess in the supernatant fluid (about 0.4 cc. per gm. of tissue). A large excess may interfere with the ribose determination later. In case the purine content of the nucleotide fraction is to be studied, 105 cc. of the protein-free filtrate should be reserved for this purpose and handled as described elsewhere (2), and the remainder treated as just mentioned in a flask or 200 cc. centrifuge bottle. After being centrifuged, the supernatant fluid and washings from *all* uranium precipitates are reserved for the subsequent study of nucleoside and free purines. An alternative procedure, recommended for autolyzed tissues with a low content of nucleotide, is to utilize the uranium precipitate from the entire filtrate. In this case the precipitate, after two washings with the diluted acidified uranium reagent, is dissolved from the walls of the flask and centrifuge bottle by means of 10 N H_2SO_4 (2 cc. for each 100 cc. of protein-free filtrate used) and collected in a single 50 cc. centrifuge tube. The usual procedure for the analysis of the nucleotide fraction is then followed (2).

In case the supernatant fluid is not perfectly clear after centrifugation, it should be filtered to insure complete removal of nucleotide before the precipitation of purines by silver nitrate.

Separation of Free Purines (Acid Silver Precipitation)—In our preliminary experiments with pure compounds the acidity produced by 0.02 N H_2SO_4 (pH 2.0) was sufficient to prevent the precipitation of nucleosides by silver nitrate. In extracts of autolyzed tissue, however, the concentration of H_2SO_4 must be increased to 0.035 or 0.045 N to bring the solution to pH

2.0, presumably because of changes occurring during autolysis. Since at least 95 per cent of free purine is precipitated by $AgNO_3$ in 0.05 N H_2SO_4 (Table I), it seems preferable to select this concentration for use with tissue filtrates in order to insure a pH between 1.5 and 2.0.

The filtrate and washings from the uranium precipitate are measured, transferred to a flask, acidified with H_2SO_4 until 0.05 N, and then treated with 0.02 volume of molar silver nitrate solution to precipitate the free purines.

The precipitate is separated within an hour to avoid the danger of acid hydrolysis of the nucleosides and the possible precipitation of nucleoside. In two experiments with a mixture of hypoxanthine and adenosine, however, the separation of purine and nucleoside was satisfactory even when the acid precipitate was allowed to stand overnight, either at room temperature or on ice. After sedimentation the precipitate is separated by repeatedly centrifuging portions of the mixture in 250 cc. centrifuge bottles, and is then shaken with water, rinsed into a 50 cc. centrifuge tube, and again centrifuged, the washings together with the supernatant fluid containing the nucleosides being reserved for analysis. A second washing is made to insure the removal of a product which interferes with the subsequent determination of ribose, this wash liquid being discarded. The precipitate, known hereafter as the "acid silver precipitate," is reserved for analysis.

Precipitation of Nucleosides (Alkaline Silver Precipitation)—To the supernatant fluid normal NaOH solution is added until the mixture is alkaline to phenol red. This precipitate, hereafter designated the "alkaline silver precipitate," contains not only the nucleosides and some silver oxide, but also whatever uranium was left in solution after removal of the nucleotides. The precipitate is collected in 250 cc. centrifuge bottles, then transferred to a 50 cc. tube while being washed twice by the same procedure as for the acid silver precipitate.

Extraction of Purines and Ribose from Silver Precipitates—From the silver precipitates the purines are extracted with HCl. The decomposition of nucleosides to their constituent purines and ribose is essential before removal of uranium and also for the subsequent precipitation of purines by copper sulfate and bisulfite. The precipitates are therefore heated in boiling water with 15 cc. of 0.5 N HCl for 30 minutes, then filtered hot with suction through asbestos in a Gooch filter mounted in a Witt filtering apparatus,[1] the extracts being received in centrifuge tubes with conical tip and graduated at 35 cc. Since AgCl adsorbs ribose strongly, it is advisable to retain as much of the precipitate as possible in the centrifuge tube in order to

[1] This is a suction flask with a ground glass removable top.

digest it repeatedly (five or six times) with 0.5 N HCl at 100°. Further washings should not give Bial's test for pentose. When cool, the filtrate is diluted to 35 cc. with 0.5 N HCl.[2]

Filter paper should not be used for filtering hot HCl if ribose is to be determined, for the hot acid extracts substances which give the pentose reaction with Bial's reagent, the amount depending on the time required for filtration as well as the quality of the paper.

The nucleoside may also be extracted as such from the silver precipitate without hydrolysis by eight extractions with cold 0.2 N HCl, and filtered through acid-washed paper (previously tested for pentose). Under these conditions the nucleoside is only slowly hydrolyzed.[3] The cold filtration is so slow, however, that we prefer the hot extraction with the Witt filter. If the cold extraction procedure is used, the nucleoside in the HCl extract must subsequently be hydrolyzed to free purine and ribose before removal of the uranium.

Analysis of HCl Extracts—Ribose must be determined before the removal of uranium, since much of it is lost in the uranium precipitate which forms when the extract is neutralized. The ribose cannot be recovered from the precipitate even if it is dissolved and reprecipitated seven times. The free purines on the other hand are easily separated by a single reprecipitation.

For the ribose determination aliquots corresponding to 1 cc. of the acid silver extract and 0.2 cc. of the alkaline silver extract are suitable. For the latter, 1 cc. of the 35 cc. of HCl extract is diluted to 10 cc., and 2 cc. of this are taken for a preliminary estimation by the method described below. The determination is repeated if necessary with an aliquot containing from 0.02 to 0.05 mg. of ribose.

Uranium does not interfere with the ribose determination unless present in excessive quantities. The volume of the centrifuged uranium precipitate formed in the next operation should be measured roughly. If the aliquot portion used for the ribose determination does not represent more than 0.15 cc. of precipitate, the ribose determination is reliable. In other words, interference will occur if the ribose determination requires a full cc. aliquot, with the volume of the precipitate exceeding 5 cc. The necessity of

[2] In the quantitative determination of ribose by Bial's reaction the acidity of the standard and unknown must be identical; hence it is convenient to keep the HCl concentration of this filtrate at a uniform level.

[3] At 25° in 0.2 N HCl only 2 per cent of adenosine was split within an hour, 13 per cent in 24 hours. At 100° the hydrolysis was complete in 40 minutes. The rate of hydrolysis was determined by measuring the liberated ribose by the method of Hagedorn and Jensen (6). The sugar equivalent of the reagent was determined on pure *d*-ribose (Pfanstiehl).

avoiding excessive amounts of uranium reagent for precipitating nucleotides is evident.

After the completion of the determination of ribose and before the precipitation of purines, uranium is precipitated from the remainder of the extract by neutralizing to phenolphthalein with 20 per cent NaOH and then discharging the indicator color with 5 per cent acetic acid. The precipitate is centrifuged and the supernatant fluid transferred without loss to a 50 cc. centrifuge tube with conical tip. In place of washing, the precipitate is dissolved in a few cc. of N H_2SO_4, then reprecipitated. The combined supernatant fluids are next heated in boiling water, the purines are precipitated by addition of copper sulfate and 40 per cent sodium bisulfite,[4] and the content of individual purines determined by methods previously described (2, 7–9).

Guanine may be determined (7) on a 3 to 5 cc. aliquot of the 25 cc. of purine hydrochloride solution secured after decomposition of the cuprous bisulfite-purine complex with H_2S. This determination, based upon the reaction with the phenol reagent, does not distinguish between guanine and xanthine. It has been shown, however, that guanine but not xanthine is found in the fresh muscle of the frog and rabbit (10–12); hence in a study to be published shortly we have designated the substance measured by Hitchings' method as guanine, with the realization that it may consist in part of xanthine, particularly in autolyzed tissue.

The presence of guanine in trichloroacetic acid extracts of tissues (7) introduces some difficulties in the determination of adenine and hypoxanthine. Hitchings[5] states that when more than 0.1 mg. of guanine N is present, some of it precipitates together with adenine when the latter is determined as picrate, the remainder being precipitated together with hypoxanthine as the argenti-picrate. In our experience with a number of tissues, the guanine content is not above the limit of 0.1 mg. of N in the nucleotide fraction when the aliquot used for the adenine determination represents 5 to 6 gm. of tissue. When the same determination is made on HCl extracts of the silver precipitates, the aliquots used are necessarily much larger, representing about 15 gm. of tissue, and the amount of guanine N greatly exceeds 0.1 mg. in some cases, particularly in autolyzed tissues. Hence it is probable that the values found for adenine in these cases are too high.

Any guanine present and not carried down with adenine as picrate would precipitate as argenti-picrate with hypoxanthine. Under the conditions defined by Hitchings for the precipitation of hypoxanthine (9) we find

[4] This reagent does not keep well. The bisulfite content was found to have decreased from 40 to 36 per cent when kept at room temperature for 10 days.

[5] Personal communication.

that each molecule of guanine combines with 1 atom of silver, as is the case with hypoxanthine.[6] Hence the amount of silver found represents the sum of hypoxanthine and guanine. Hypoxanthine is therefore determined by subtracting the guanine (determined colorimetrically) from total silver, each calculated as moles, since the nitrogen contents of hypoxanthine and guanine differ. If any guanine were precipitated with adenine, this method of calculation for hypoxanthine by difference would result in values which are too low (or even negative if no hypoxanthine were present), as the amount of guanine determined in the original mixture of the purines was greater than that precipitated as argenti-picrate.

At present we have no solution to offer for these difficulties, but wish to call attention to the sources of error which are encountered chiefly in experiments with autolyzed tissues.

Determination of Ribose—Dische and Schwartz (13) and Mejbaum (14) stated the conditions under which Bial's reaction for pentoses (15) gives an intensity of color proportional to concentration, the colors being measured in the Pulfrich spectrophotometer with Filter S-61. We have made certain modifications in order to permit the use of an ordinary colorimeter.

Mejbaum (14) noted that the intensity of color is proportional to concentration only with very dilute solutions of pentose, and this we have confirmed on attempting to use larger samples.

A series of experiments to determine the optimum concentrations of HCl, $FeCl_3$, and orcinol showed that the intensity of color produced on heating pentoses with these reagents is equally great with much lower concentrations of $FeCl_3$ and orcinol than those used by other workers (13–15). The use of the higher concentration of $FeCl_3$ not only gives a deeper yellow color to the reagent, but also an increase in intensity which affects the colorimeter reading when the reagents are heated as a blank without pentose.

As the color production is particularly sensitive to the concentration of HCl, *it is essential that the unknown and standard have exactly the same acidity during the period of heating.* An error of +25 per cent results when the unknown contains 5.25 N HCl and the standard 5 N. In the procedure outlined above for tissue analysis pentose is determined in the 0.5 N HCl extract of the silver precipitate; hence extra acid must be added to the standard to compensate for that in the sample analyzed.

Reagents—

Concentrated HCl containing 0.02 per cent $FeCl_3$.

Orcinol, 10 per cent solution in ethyl alcohol. This should be nearly colorless when freshly prepared and not darkened when stored in a stoppered vessel.

[6] Uupublished data.

Standard pentose solution. A solution of *d*-ribose (Pfanstiehl, dehydrated over sulfuric acid *in vacuo*) in 0.1 N HCl containing 1.0 mg. per 100 cc. (3 cc. = 0.03 mg.). *d*-Arabinose may be substituted for ribose.

Procedure

Measure into a test-tube graduated at 15 cc. a quantity of the unknown solution containing between 0.02 and 0.05 mg. of ribose. Add 0.3 cc. of N HCl (the HCl content of the standard ribose solution) and dilute to 5 cc.

Prepare the standard by measuring into a similar tube 3 cc. of the standard ribose solution (0.03 mg. of ribose), also a quantity of N HCl equal to that found in the unknown sample taken, and dilute to 5 cc.

To both the standard and unknown add 5.0 cc. of the HCl-$FeCl_3$ reagent and 0.3 cc. of orcinol solution and mix. Immerse the tubes in boiling water for 20 minutes, then cool, dilute to 15 cc., and compare in the colorimeter, using a Wratten Filter E-22 in the light path.[7] The color is stable for hours.

The results obtained with varying concentrations of pentose in the form of pure *d*-ribose, *d*-arabinose, adenosine, and myoadenylic acid are given in Table IV. Arabinose gives the same intensity of color as ribose, and may therefore be substituted as standard. The ribose bound in adenosine is correctly measured by this reaction, but in the case of myoadenylic acid with all concentrations used the ribose found is 20 per cent higher than the calculated content.

A more rapid development of color may be secured by using 10 cc. of reagent and 5 cc. of unknown, thus giving an acid concentration of 6.7 N. Under these conditions the color intensity after 5 minutes heating is equal to that with 5 N HCl as recommended in our procedure, but we have had more consistent results with the lower concentration of acid and longer heating, probably because of the slower liberation of furfural. For qualitative tests, however, the best results are obtained by heating the unknown for 5 minutes with 2 volumes of concentrated HCl containing only 0.008 per cent $FeCl_3$ and 0.3 per cent orcinol. The lower concentration of iron gives an almost colorless reagent and permits the detection of smaller amounts of pentose.

Interference by Chlorides—Some adenosine is carried down with the acid silver precipitate when chlorides are present, the amount being dependent on the chloride concentration. In various beef tissues chlorides, calculated as NaCl, range in concentration from a minimum of 0.08 per cent in

[7] These filters may be procured from the Eastman Kodak Company in disks suitable for use in the eyepiece of a colorimeter, or squares to be inserted between the light source and the colorimeter.

skeletal muscle to 0.44 per cent in kidney (16). Hence in trichloroacetic acid extracts of tissues with a 10-fold dilution the concentration of NaCl is always less than 0.05 per cent.

A number of experiments were made to determine the amount of nucleoside carried down with AgCl at an acidity of 0.05 N H_2SO_4. With 25 cc. samples containing 0.10 per cent NaCl and adenosine equivalent to 0.08, 0.32, and 0.86 mg. of ribose, the adsorption of ribose on the acid silver precipitate amounted to 0.018, 0.012, and 0.023 mg. respectively, the loss

TABLE IV

Determinations of Ribose, Free and Combined, in Adenosine and Adenylic Acid by Modified Bial's Reaction; All Determinations Made in Colorimeter with Wratten Filter E-22 and 0 03 Mg of Ribose As Standard

Compound	Pentose taken	Pentose found	Recovery
	mg	*mg*	*per cent*
d-Ribose	0 0209	0 0216	103 4
"	0 0418	0 0418	100 0
"	0 0523	0 0532	101 8
d-Arabinose	0 03	0 0306	103 0
"	0 05	0 0515	103 0
"	0 05	0 0503	100 6
Adenosine	0 0247	0 0239	96 8
"	0 0371	0 0361	97 2
"	0 0494	0 0492	99 6
"	0 0618	0 0006	98 1
Guanosine	0 0233	0 0229	98 5
"	0 0529	0 0538	101 9
Myoadenylic acid	0 0242	0 0293	121 1
" "	0 0363	0 0432	119 0
" "	0 0484	0 0588	121 5
" "	0 0605	0 0732	121 0
Ba ribose monophosphate	0 0173	0 0218	126 0
" " "	0 0288	0 353	122 8

being unrelated to the amount of nucleoside in the sample. The concentration of chloride chosen for these experiments is, however, 10 times that found in muscle filtrates, at least double that found in any beef tissue filtrate (1:10 dilution), and is encountered only in blood filtrates with a 1:5 dilution.

Recovery of Added Purine and Nucleoside—The ability to recover added purine and nucleoside by the above methods was tested by analyzing duplicate portions (400 cc. each) of trichloroacetic extracts of dog brain, to one of which known amounts of adenine and adenosine were added. The results, presented in Table V, indicate that the separation of purine from

nucleoside is not quite as sharp when applied to tissue extracts as with pure solutions, a small amount of nucleoside being carried down with the acid silver precipitate, probably because of the presence of chlorides. The results are sufficiently good, however, to justify the use of the method for studies on the nucleotide and purine metabolism of tissues.

Since the greatest interference is to be expected in the analysis of blood, because of its relatively high content of chloride, three experiments were made to determine the extent of recovery of adenosine added to dog blood.

When 0.85 mg. of adenosine N was added per 100 cc. of blood, and specimens of 100 to 150 cc. used for analysis, the recovery of adenosine in the alkaline silver precipitate was 80.5 and 84.4 per cent. When the adenosine added was reduced to 0.17 mg. of N per 100 cc., the recovery was 70 per cent.

TABLE V

Recovery of Adenine and Adenosine Added to Trichloroacetic Acid Extracts of Brain

Added		Recovered in acid Ag ppt.		Recovered in alkaline Ag ppt.	
		mg.	*per cent*	*mg.*	*per cent*
Adenine	3.08 mg. N	3 14	102		
Adenosine	3 82 " "			3 66	95.8
	8 20 " ribose	0.33	4	7.1	86.5
Adenine	3.22 " N	3.22	100		
Adenosine	3.82 " "			3.41	89.3
	8.20 " ribose	0		7.47	90.8

Attempts to recover adenosine added to urine have so far been unsuccessful.

DISCUSSION

Silver in ammoniacal solution has long been used as a precipitant for the purine bases (17–19), but we find it does not precipitate adenosine or adenylic acid, and Pohle (20) reported the same for inosinic acid. Substitution of NaOH for ammonia gives the silver reagent the property of a general precipitant for the purine nucleotides and their derivatives (myoadenylic acid, adenosine, inosine, guanosine, adenine, hypoxanthine, and guanine having been tested). In acid solution, however, only the free purines are precipitated by silver nitrate, at least in the concentrations encountered in extracts of fresh and autolyzed tissue.[8] Similarly, basic

[8] Our studies were carried out with concentrations of nucleoside and free purine slightly higher than any encountered in tissue analysis. The highest concentration of adenosine we have found in any autolyzed tissue was 11.2 mg. of N per 100 gm.,

lead acetate or lead acetate and ammonia, but not neutral or acid solutions of lead acetate, precipitate the nucleosides (21, 22).

Bielschowsky (23) attempted to separate nucleosides from solution by means of mercuric acetate, but stated the precipitation to be incomplete. Inagaki (24) also noted that the purine nucleosides are only partially precipitated by mercuric acetate and sulfate at room temperature, precipitation being complete at 0° with these reagents, or at room temperature with mercuric nitrate.

Bial's reaction for pentoses has been adapted for use in the spectrophotometer or photoelectric colorimeter by several workers (13, 14, 25, 26). The various procedures differ in some details, and this may explain the differences reported for the behavior of arabinose and ribose, and of ribose, free and combined. Dische and Schwartz (13) stated that adenosine gives a greater color intensity than myoadenylic acid, whereas we find the reverse to be true (Table IV). Mejbaum (14) found no difference in the color yielded by the pentose in the form of arabinose, myoadenylic acid, and inosinic acid. Schlenk (25) found that ribose, xylose, and arabinose give identical results when used as standards. Stone (26) considers *d*-arabinose unsuitable for use as a standard for measuring the purine nucleotides, its rate of color production being much slower than that of the ribose in adenosine triphosphate. We, too, find that myoadenylic acid gives more color than ribose or arabinose. The selection of a standard should of course be governed by the compound to be measured, arabinose and ribose being suitable for measuring nucleosides or free ribose, whereas myoadenylic acid or a salt of adenosine triphosphate of known purity should serve best for measuring nucleotides.

Barrenscheen and Peham (27) describe a photometric method for estimating nucleosides and nucleotides based on the orcinol reaction, with the substitution of cupric chloride for ferric chloride. Decreasing intensity of color was found for the pentose in adenosine triphosphate, adenosine, xylose, and arabinose.

In a study of nucleotide catabolism in various tissues[9] with the procedures described in this paper, a small amount of pentose (3 to 5 mg. per 100 gm.) was found in the acid silver precipitate, together with hypoxanthine and guanine. Some organic phosphorus was also found in this precipitate with most (but not all) tissues. The possibility that small amounts

which corresponds to 0.28 mg. of N in 25 cc. of protein-free filtrate. This was the final volume of solution used in each of the experiments reported. Adenosine in a concentration 7 times as great as this was not precipitated by $AgNO_3$ at pH 2 (see Table I). The highest concentration of inosine found in tissues was 13.2 mg. of N per 100 gm., or 0.33 mg. per 25 cc.

[9] To be published shortly.

of nucleotide escape precipitation by uranium was suggested by Parnas (11) and Ostern (12) in explanation of the adenine they found in the fraction containing nucleosides and free purines. That this is not the case is demonstrated by the fact that in the analysis of certain tissues (*e.g.*, testes) no phosphorus was found in either the acid or the alkaline silver fractions. In the case of skeletal muscle the amount of phosphorus was too little to account for the pentose in each of the silver precipitates. Ribose phosphate, if present, should be precipitated by uranyl acetate together with inorganic phosphate and nucleotide (24). We find that free ribose is not precipitated by silver nitrate and NaOH, but is adsorbed to some extent by AgCl; hence it would be found partly with the acid silver precipitate if it occurred in tissue extracts.

SUMMARY

The free purines adenine, guanine, and hypoxanthine are quantitatively precipitated by silver nitrate in the presence of sodium trichloroacetate and H_2SO_4 (0.02 to 0.05 N), whereas in *dilute* solution the nucleosides adenosine, guanosine, and inosine remain unprecipitated. All of these purines and nucleosides are quantitatively precipitated by silver nitrate when the solution is made slightly alkaline with NaOH.

A method of analysis for the nucleosides and free purines in trichloroacetic acid extracts of tissues is described. After removal of nucleotides by precipitation with uranium acetate, the free purines are precipitated by $AgNO_3$ in acid solution (pH between 1.5 and 2.0), and the nucleosides by $AgNO_3$ in the presence of a slight excess of NaOH. The purines and nucleosides are extracted from the silver precipitates by HCl and analyzed by methods previously described.

A modification of Bial's reaction for pentose is described which permits the determination of *d*-ribose in an ordinary colorimeter with a special light filter.

BIBLIOGRAPHY

1. Kerr, S. E., and Blish, M. E., *J. Biol. Chem.*, **98**, 193 (1932).
2. Kerr, S. E., *J. Biol. Chem.*, **132**, 147 (1940).
3. Kerr, S. E., *J. Biol. Chem.*, **145**, 647 (1942).
4. Kerr, S. E., *J. Biol. Chem.*, **110**, 625 (1935).
5. Thannhauser, S. J., and Czoniczer, G., *Z. physiol. Chem.*, **110**, 307 (1920).
6. Hagedorn, H. C., and Jensen, B. N., *Biochem. Z.*, **135**, 46 (1923); **137**, 92 (1923).
7. Hitchings, G. H., *J. Biol. Chem.*, **139**, 843 (1941).
8. Hitchings, G. H., and Fiske, C. H., *J. Biol. Chem.*, **140**, 491 (1941); **141**, 827 (1941).
9. Hitchings, G. H., *J. Biol. Chem.*, **143**, 43 (1942).
10. Dmochowski, A., *Biochem. J.*, **23**, 1346 (1929).
11. Parnas, J. K., *Biochem. Z.*, **206**, 16 (1929).
12. Ostern, P., *Biochem. Z.*, **221**, 64 (1930).

13. Dische, Z., and Schwartz, K., *Mikrochim. acta*, **2**, 13 (1938).
14. Mejbaum, W., *Z. physiol. Chem.*, **258**, 117 (1939).
15. Bial, M., *Deut. med. Wochschr.*, **28**, 252 (1902); *Berl. klin. Wochschr.*, **40**, 405 (1903).
16. Sunderman, F. W., and Williams, P., *J. Biol. Chem.*, **102**, 279 (1933).
17. Neubauer, *Z. anal. Chem.*, **6**, 33 (1867).
18. Schindler, S., *Z. physiol. Chem.*, **13**, 432 (1889).
19. Salkowski, E., *Arch. ges. Physiol.*, **69**, 280 (1898).
20. Pohle, K., *Z. physiol. Chem.*, **185**, 9 (1929).
21. Jones, W., Nucleic acids, their chemical properties and physiological conduct, Monographs on biochemistry, London and New York, 2nd edition, 111 (1920).
22. Levene, P. A., and Bass, L. W., Nucleic acids, American Chemical Society monograph series, New York, 167 (1931).
23. Bielschowsky, F., *Z. physiol. Chem.*, **190**, 15 (1930).
24. Inagaki, T., *J. Biochem.*, Japan, **32**, 57 (1940).
25. Schlenk, F., *J. Biol. Chem.*, **146**, 619 (1942).
26. Stone, W. E., *J. Biol. Chem.*, **149**, 29 (1943).
27. Barrenscheen, H. K., and Peham, A., *Z. physiol. Chem.*, **272**, 81 (1941); cited by *Chem. Abstr.*, **37**, 3024 (1943).

THE MICROBIOLOGICAL DETERMINATION OF NICOTINIC ACID, NICOTINAMIDE, AND NICOTINURIC ACID

BY B. CONNOR JOHNSON

(*From the Division of Animal Nutrition, University of Illinois, Urbana*)

(Received for publication, March 19, 1945)

The nicotinic acid assay most generally satisfactory is the *Lactobacillus arabinosus* procedure (1, 2) as regards sensitivity and non-interference from other compounds. However, this organism responds to nicotinamide and nicotinuric acid as well as to nicotinic acid.

Gaines and Stahly (3) have proposed the use of *Leuconostoc mesenteroides* for the assay of several members of the vitamin B complex, including nicotinic acid. We have found that this organism responds to nicotinic acid, but not to nicotinamide at low concentrations, nor to nicotinuric acid.

By the use of acid hydrolysis of the amide, these two organisms can be used for the determination of all three compounds.

EXPERIMENTAL

Determination of Free Nicotinic Acid—The test organism *Leuconostoc mesenteroides* (American Type Culture Collection, No. 9135) is carried on yeast extract 1 per cent, milk peptone (Difco) 1 per cent, glucose 1 per cent, agar 1.5 per cent stabs, and is transferred every 2 weeks.

The inoculum is prepared by transfer from this stab to 10 cc. of the diluted basal medium containing 1 γ of nicotinic acid. After 24 hours incubation, the cells are centrifuged out and resuspended in 10 cc. of sterile 0.9 per cent saline. 1 drop of this suspension is used per tube for inoculation.

The basal medium used is given in Table I. This medium is double strength, 5 cc. being used per tube, diluted to 10 cc. with the solution being assayed plus water. The addition of the purines to the medium of Gaines and Stahly (3) doubled the acid production, increasing the maximum titration from 3 up to 6 cc. By the increase in buffer and glucose and other minor changes, this medium now gives a maximum titration of 12 to 15 cc. of 0.1 N NaOH per tube.

It was found that this organism, as in the case of *Lactobacillus casei* and *Streptococcus lactis*, utilizes "pseudopyridoxine" rather than pyridoxine (5). This can be supplied either by pyridoxine which has been treated with hydrogen peroxide (6) or by pyridoxal (7). The organism responds to the thiazole moiety of thiamine as well as to intact thiamine. For these reasons it cannot be used for pyridoxine or thiamine assays as proposed by Gaines and Stahly (3).

The assay is set up in the same manner as the *Lactobacillus arabinosus* assay of Snell and Wright (1). The tubes are incubated at 30° for 72 hours and the acid formed is titrated with 0.1 N NaOH.

At levels of acid production above about 8 cc. of 0.1 N acid per tube, this organism produces a considerable amount of CO_2 which interferes with the end-point of the titration. This can readily be eliminated by autoclaving the tubes and cooling them before titration.

On Fig. 1 is plotted the dose-response curve of this organism to nicotinic acid, nicotinamide, and nicotinuric acid. While there is no increase in acid production with the addition of nicotinamide or nicotinuric acid up

TABLE I

Basal Medium

Acid-hydrolyzed vitamin-free casein	10 gm.
Glucose	60 "
Sodium acetate, anhydrous	40 "
Cystine	20 mg.
l-Tryptophane	10 "
Salt Solution A*	10 cc.
" " B*	10 "
Riboflavin	500 γ
Thiamine	500
Calcium pantothenate	500
Biotin	10
"Pyridoxal"	100
Adenine	20 mg
Guanine	20
Xanthine	20
Asparagine	50
p-Aminobenzoic acid	100 γ
Distilled water to	1000 cc.

* Prepared according to Snell and Strong (4)

to 10 γ per tube, which is about the maximum for nicotinic acid assay, nicotinamide does have some slight growth effect and at 1000 γ per tube an acid production equal to 8 cc. of 0.1 N NaOH per tube was obtained.

Determination of Nicotinamide—In order to determine nicotinamide with *Leuconostoc mesenteroides*, it must first be hydrolyzed to nicotinic acid by a procedure which gives practically complete hydrolysis of the amide and at the same time avoids appreciable hydrolysis of nicotinuric acid if this is present. Hydrochloric acid (8) has been used. However, it was found that the amount of NaCl formed on neutralization inhibited the growth of the bacteria. Therefore, sulfuric acid was used. Solutions containing 1 γ per cc. of nicotinamide or of nicotinuric acid were autoclaved for

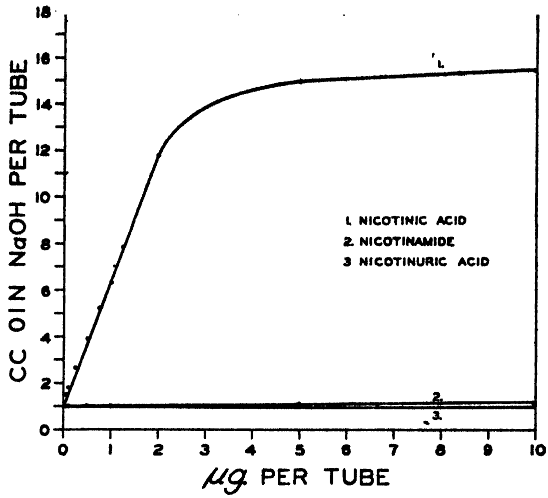


FIG. 1. Dose-response of nicotinic acid, nicotinamide, and nicotinuric acid with *Leuconostoc mesenteroides*.

TABLE II

Sulfuric Acid Hydrolysis of Nicotinamide and Nicotinuric Acid

Normality of acid	Per cent hydrolysis	
	Nicotinamide	Nicotinuric acid
0.0	0	0
0.1	21	
0.2	34.5	
0.5	80	
0.6	100	0
0.8	100	0
1.0	100	0
6.0		83

TABLE III

Recovery of Known Amounts of Nicotinic Acid and Its Derivatives

The material assayed was a water solution containing 0.2 γ per cc. each of nicotinic acid, nicotinamide, and nicotinuric acid.

	Sample No.	No. of tests*	Treatment	Found as nicotinic acid	Recovery
				γ	*per cent*
Leuconostoc mesenteroides	1	2	None	0.20	100
	2	4	0.6 N H_2SO_4 hydrolysis	0.38	95
Lactobacillus arabinosus	3	2	None	0.58	96.2

* Each test was run at five levels of assay with duplicate tubes at each level.

1 hour at 15 pounds with different strengths of sulfuric acid. They were then cooled, neutralized with saturated $Ba(OH)_2$ solution, diluted to 0.2 γ per cc., filtered, and assayed. The relationship between the strength of the acid and per cent hydrolysis for the two compounds is given in Table II.

From Table II it can be seen that 0.6 N sulfuric acid completely hydrolyzes nicotinamide in 1 hour at 15 pounds and at the same time causes practically no hydrolysis of nicotinuric acid. Therefore the difference between the assay value obtained with *Leuconostoc mesenteroides* before acid hydrolysis with 0.6 N H_2SO_4 and that obtained after acid hydrolysis represents the amount of nicotinamide that has been converted to free nicotinic acid.

Determination of Nicotinuric Acid—We have determined nicotinuric acid as the difference between the total nicotinic acid obtained with *Lactobacillus arabinosus* by the method of Krehl, Strong, and Elvehjem (2) and that obtained with *Leuconostoc mesenteroides* after 0.6 N H_2SO_4 hydrolysis.

Average data obtained on the analysis of pure solutions of nicotinic acid, nicotinamide, and nicotinuric acid by the procedure finally adopted are given in Table III. Similar data obtained on human urine substantiate these results and are given in the following paper (Johnson, Hamilton, and Mitchell (9)).

SUMMARY

A method for the determination of nicotinic acid, nicotinamide, and nicotinuric acid in solution containing one or more of these compounds is proposed.

The method is based on the finding that the organism, *Leuconostoc mesenteroides* which requires nicotinic acid for growth, is unable to use nicotinamide or nicotinuric acid at the levels of assay investigated.

BIBLIOGRAPHY

1. Snell, E. E., and Wright, L. D., *J. Biol. Chem.*, **139**, 675 (1941).
2. Krehl, W. A., Strong, F. M., and Elvehjem, C. A., *Ind. and Eng. Chem., Anal. Ed.*, **15**, 471 (1943).
3. Gaines, S., and Stahly, G. L., *J. Bact.*, **46**, 441 (1943).
4. Snell, E. E., and Strong, F. M., *Ind. and Eng. Chem., Anal. Ed.*, **11**, 346 (1939).
5. Johnson, B. C., *Proc. Soc. Exp. Biol. and Med.*, **55**, 199 (1944).
6. Carpenter, L. E., and Strong, F. M., *Arch. Biochem.*, **3**, 375 (1944).
7. Snell, E. E., *J. Biol. Chem.*, **154**, 313 (1944).
8. Melnick, D., Robinson, W. D., and Field, H., Jr., *J. Biol. Chem.*, **136**, 145 (1940).
9. Johnson, B. C., Hamilton, T. S., and Mitchell, H. H., *J. Biol. Chem.*, **159**, 231 (1945).

THE EXCRETION OF NICOTINIC ACID, NICOTINAMIDE, NICOTINURIC ACID, AND N[1]-METHYLNICOTINAMIDE BY NORMAL INDIVIDUALS*

By B. CONNOR JOHNSON, T. S. HAMILTON, and H. H. MITCHELL

(*From the Division of Animal Nutrition, University of Illinois, Urbana*)

(Received for publication, March 19, 1945)

The metabolites of nicotinic acid excreted in urine have been studied by a number of workers. The occurrence of a nicotinic acid and amide fraction, of nicotinuric acid, and of N[1]-methylnicotinamide (or F_2), or trigonelline in urine has been reported. Melnick, Robinson, and Field (1) did not find nicotinuric acid unless large doses of nicotinic acid (as distinguished from the amide) were fed. Perlzweig (2) and Sarett (3, 4), however, later reported the presence of nicotinuric acid in normal human urine. Using the *Leuconostoc mesenteroides* method described in the preceding paper, we have been unable to find nicotinuric acid in human urine.

No work has been reported on the separate determinations of nicotinic acid and nicotinamide excreted in the urine. The bacteriological method which we have developed has enabled us to make these separate determinations.

EXPERIMENTAL

Four male subjects aged 21 to 28 years were used in this work. They were on a constant diet throughout the preliminary and experimental periods, during which time their activity was entirely sedentary.

The experiment was divided into two 5 day periods. During Period I no nicotinic acid was given in addition to that contained in the diet. Each subject received orally 50 mg. per day of nicotinamide during Period II.

The 24 hour urine specimens were collected each day and combined for the 5 day period. 5 per cent acetic acid was used as a preservative.

Analyses were carried out for total nicotinic acid (*i.e.*, nicotinic acid, nicotinamide, and nicotinuric acid) by the *Lactobacillus arabinosus* microbiological assay procedure of Snell and Wright (5) as modified by Krehl *et al.* (6). The same urine samples were also analyzed for these individual constituents by the *Leuconostoc mesenteroides* procedure described in the preceding paper. The N[1]-methylnicotinamide determinations were carried out by the fluorometric procedure of Huff and Perlzweig (7).

* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Illinois.

The urinary concentration values were found to confirm the findings of other workers that N^1-methylnicotinamide is excreted in higher concentration than the total of all other known metabolites of nicotinic acid.

A 65 per cent increase in nicotinic acid concentration in urine following the administration of 50 mg. of nicotinamide daily with no concomitant rise in nicotinamide was noted and may be significant. In agreement with the report of Melnick *et al.* (1), we were unable to find a significant concentration of nicotinuric acid in the urine. However, because of the positive findings of other workers and in order to establish further the validity of our assay procedure, the following experiment was carried out.

Nicotinuric acid was added to water, to urine, to urine followed by 0.6 N H_2SO_4 hydrolysis, and to urine followed by 6 N H_2SO_4 hydrolysis. In

TABLE I

Recovery of Nicotinuric Acid

Sample No.	Sample	Nicotinuric acid added	H_2SO_4 hydrolysis	*Leuconostoc mesenteroides* assay	
				Free nicotinic acid	Recovery of nicotinuric acid
		γ *per cc.*	N	γ *per cc.*	*per cent*
1	Water	1		0	0
2	"	1	0.6	0.017	1.7
3	Urine	0		0.35	
4	"	0	0.6	0.925	
5	"	1		0.348	0
6	"	1	0.6	0.93	0
7	"	0	6	0.93	
8	"	1	6	1.76	83

the preceding paper it was shown that 0.6 N H_2SO_4 completely hydrolyzes nicotinamide but does not hydrolyze nicotinuric acid in water solutions. However, there was the possibility that nicotinuric acid might be hydrolyzed by 0.6 N H_2SO_4 in the presence of urine. 6 N acid was used in order to insure some hydrolysis of nicotinuric acid so that an added amount could be partially recovered. From the data in Table I, it is evident that practically no hydrolysis of nicotinuric acid occurs with 0.6 N H_2SO_4 even in the presence of urine. The data also indicate the absence of nicotinuric acid, since 6 N H_2SO_4 hydrolysis gave no increase in free nicotinic acid over that obtained with 0.6 N H_2SO_4, while added nicotinuric acid was largely recovered (83 per cent) after 6 N H_2SO_4 hydrolysis.

Table II gives the total daily excretion of nicotinic acid and its metabolites. There is a small increase in nicotinic acid excretion following nicotinamide dosage. It appears from Table II that 94 to 95 per cent of

the nicotinic acid excreted is in the form of N^1-methylnicotinamide and only 5 to 6 per cent is excreted as the free acid and amide.

Our failure to find any large immediate increase in the excretion of N^1-methylnicotinamide following daily 50 mg. dosage of nicotinamide is in agreement with the work of Mickelsen (8) who found no increase in F_2 excretion following 10 mg. doses of nicotinic acid.

During the experimental periods, the subjects were maintained 8 hours per day in a hot environment of 37.8° and 70 per cent relative humidity.

TABLE II

Daily Excretion of Nicotinic Acid and Its Metabolites; Estimated Daily Nicotinic Acid Plus Amide Intake

Period No.	Subject	Urine volume per day	Free nicotinic acid	Nicotinamide	N^1-Methyl nicotinamide	Total	Estimated intake*
		cc.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
I. No dosage	C	850	0.17	0.70	10.8	11.67	19.0
	D	1616	0.40	1.28	42.3	44.0	30.0
	E	990	0.21	0.80	8.5	9.5	15.3
	F	1130	0.17	0.87	13.1	14.1	20.1
Average			0.24	0.91	18.7	19.8	21.1
% of total			1.2	4.6	94.4	100	
II. 50 mg. nicotinamide per day	C	754	0.24	0.56	11.0	11.8	19.0 + 50
	D	1484	0.50	1.15	39.8	41.5	30.0 + 50
	E	852	0.34	0.56	14.0	14.9	15.3 + 50
	F	967	0.25	0.73	15.9	16.9	20.1 + 50
Average			0.33	0.75	20.2	21.3	71.1
% of total			1.5	3.5	94.8	100	

* These estimates are made from the lower range of published values obtained by the microbiological assay method of Snell and Wright (5).

The total skin excretion was collected for each 8 hour period. In order to determine the concentration of nicotinic acid and its metabolites in sweat, undiluted sweat was collected from each subject during 4 hour periods on the days following the end of each 5 day period. The sweat samples were stored and analyzed in the same manner as the urine samples. Table III summarizes the concentration data obtained for total nicotinic acid, free nicotinic acid, nicotinamide, and N^1-methylnicotinamide in undiluted sweat. As in the case of urine, no nicotinuric acid was found in sweat.

A summary of the daily skin excretion data on the 5 day collection

TABLE III

Concentration of Nicotinic Acid and Its Metabolites in Sweat

The values are given in micrograms per cc.

Period preceding collection of undiluted sweat	Subject	Total nicotinic acid (*Lactobacillus arabinosus*)	Free nicotinic acid (*Leuconostoc mesenteroides*)	Nicotinamide	N^1-Methylnicotinamide	Total
I. No dosage	C	0.046	0.035	0.011		
	D	0.096	0.087	0.009		
	E	0.031	0.017	0.013	0.059	0.089
	F	0.032	0.029	0.003	0.037	0.069
Average		0.051	0.042	0.009	0.048	0.079
% of total*			30	10	60	100
II. 50 mg. nicotinamide per day	C	0.033	0.033	0.000	0.060	0.093
	D	0.064	0.060	0.004	0.159	0.223
	E	0.064	0.050	0.014	0.048	0.112
	F	0.047	0.049	0.000	0.090	0.137
Average		0.052	0.048	0.004	0.089	0.141
% of total			34	3	63	100

* Based on Subjects E and F only.

TABLE IV

8 Hour Excretion of Nicotinic Acid and Its Metabolites Through the Skin

The values are given in mg.

Period No.	Subject	Nicotinic acid	Nicotinamide	N^1-Methylnicotinamide	Total
I. No dosage	C	0.097	0.025	0.268	0.390
	D	0.160	0.022	0.345	0.527
	E	0.109	0.031	0.371	0.511
	F	0.116	0.024	0.407	0.547
Average		0.120	0.025	0.348	0.494
% of total		24.3	5.2	70.5	100
II. 50 mg. nicotinamide per day	C	0.080	0.046	0.299	0.425
	D	0.146	0.037	0.332	0.515
	E	0.168	0.060	0.365	0.594
	F	0.137	0.041	0.308	0.486
Average		0.133	0.046	0.326	0.505
% of total		26.3	9.1	64.6	100

periods is given in Table IV, from which it appears that the amounts of nicotinic acid and its metabolites that would be excreted in sweat under conditions inducing profuse sweating throughout the day (*i. e.*, data in

Table IV multiplied by 3 to convert them to the 24 hour basis) are too small to have any significant influence on the nicotinic acid requirements of persons subjected to a hot environment. It is interesting to note, however, that again N^1-methylnicotinamide is the chief metabolite excreted. This is in contrast to the work of Sargent, Robinson, and Johnson (9) who found none. In sweat a higher percentage of the nicotinic acid excreted was present as the free acid and less as N^1-methylnicotinamide than in the case of urine.

DISCUSSION

The results of these experiments confirm the findings of other workers that the principal excretory product of nicotinic acid metabolism is N^1-methylnicotinamide which in our work amounted to over 94 per cent of the total excretion of nicotinic acid and metabolites in the urine.

The 50 per cent increase in the excretion of free nicotinic acid after feeding nicotinamide was proved to be statistically significant (probability of less than 0.0069 by the method of "Student" (10)) and suggests that some of the nicotinamide fed is deaminated to free nicotinic acid and excreted as such. Following 50 mg. daily doses of nicotinamide during the second experimental period, an average increase of only 1.5 mg. per day (maximum 5.4) was excreted as N^1-methylnicotinamide. Thus only about 3 per cent of the ingested nicotinamide was recovered in the urine, pointing to a large destruction and also possibly to storage in some form. Najjar and coworkers (11) have shown that there is a considerable capacity for storage of N^1-methylnicotinamide in the dog.

The absence of nicotinuric acid in the urine supports the view that nicotinuric acid excretion occurs only as a detoxication product of nicotinic acid when the latter is fed in large doses. The vasodilatory effect of nicotinic acid is well known and is not exhibited by nicotinamide or nicotinuric acid. Melnick *et al.* (1) observed that nicotinuric acid was excreted in considerable amounts after 500 mg. doses of nicotinic acid.

SUMMARY

1. The excretion of nicotinic acid, nicotinamide, nicotinuric acid, and N^1-methylnicotinamide in the urine and sweat of four men subjected to a hot moist environment was studied.

2. Over 94 per cent of the total nicotinic acid and metabolites excreted in the urine was in the form of N^1-methylnicotinamide.

3. 1 to 1.5 per cent was excreted as free nicotinic acid and 3.5 to 4.5 per cent as nicotinamide.

4. No nicotinuric acid was found in either urine or sweat.

5. The amounts of nicotinic acid and its metabolites present in sweat are too small to have any significant influence on the nicotinic acid re-

quirement of persons subjected to an environment which induces profuse sweating throughout the day.

BIBLIOGRAPHY

1. Melnick, D., Robinson, W. D., and Field, H., Jr., *J. Biol. Chem.*, **136**, 145 (1940).
2. Perlzweig, W. A., Levy, E. D., and Sarett, H. P., *J. Biol. Chem.*, **136**, 729 (1940).
3. Sarett, H. P., Huff, J. W., and Perlzweig, W. A., *J. Nutr.*, **23**, 23 (1942).
4. Sarett, H. P., *J. Nutr.*, **23**, 35 (1942).
5. Snell, E. E., and Wright, L. D., *J. Biol. Chem.*, **139**, 675 (1941).
6. Krehl, W. A., Strong, F. M., and Elvehjem, C. A., *Ind. and Eng. Chem., Anal. Ed.*, **15**, 471 (1943).
7. Huff, J. W., and Perlzweig, W. A., *J. Biol. Chem.*, **150**, 483 (1943).
8. Mickelsen, O., *Federation Proc.*, **3**, 61 (1944).
9. Sargent, F., Robinson, P., and Johnson, R. E., *J. Biol. Chem.*, **153**, 285 (1944).
10. "Student," *Biometrika*, **6**, 1 (1908).
11. Najjar, V. A., Hammond, M. M., English, M. A., Wooden, M. B., and Deal, C. C., *Bull. Johns Hopkins Hosp.*, **74**, 406 (1944).

LETTERS TO THE EDITORS

GROWTH STIMULANTS IN THE LACTOBACILLUS ARABINOSUS BIOTIN ASSAY

Sirs:

It has recently been reported[1] that in the microbiological assay of biotin certain lipoidal substances act as growth stimulants for *Lactobacillus casei*.

Apparent Biotin Content of Rice Polish Samples Corresponding to Different Concentrations of Extract

Test organism	Rice polish extract per tube	Method of extract preparation		
		Simple hydrolysis*	Hydrolysis and filtration†	Hydrolysis of defatted rice polish‡
	ml.	*γ per gm.*	*γ per gm.*	*γ per gm.*
Lactobacillus casei	1.00	0.96	0.38	
	2.00	0.76	0.36	
		0.70		0.32
	3.00	0.73	0.33	
		0.56		0.36
Lactobacillus arabinosus	1.00	0.62	0.34	
	2.00	0.57	0.34	
		0.52		0.36
	3.00	0.74	0.32	
		0.46		0.34

* Rice polish extract prepared by hydrolyzing 0.2500 gm. of rice polish in 50 ml. of normal sulfuric acid for 30 minutes at 15 pounds pressure. The pH was adjusted to 6.6 to 6.8 and the sample diluted to 1 liter.

† Hydrolysate prepared as described above and filtered through Whatman ashless fine filter paper.

‡ Rice polish extracted with petroleum ether for 8 hours in a Soxhlet extractor prior to hydrolysis. Weight corrected for removal of fat.

Previous investigators had observed similar effects in the assay of pantothenic acid and riboflavin with the same organism.[2] It is now found that

[1] Williams, V. R., and Fieger, E. A., *Cereal Chem.*, **21**, 540 (1944); *Ind. and Eng. Chem., Anal. Ed.*, **17**, 127 (1945).

[2] Strong, F. M., and Carpenter, L. E., *Ind. and Eng. Chem., Anal. Ed.*, **14**, 909 (1942).

the assay of biotin with *Lactobacillus arabinosus* is similarly sensitive to lipid growth stimulants present in rice polish, and the failure to remove such stimulants prior to assay leads to anomalies, as is shown in the table. An investigation of the nature of the lipid substances in rice polish producing the stimulation is being continued.

Department of Agricultural Chemistry and Biochemistry
Louisiana Agricultural Experiment Station
Louisiana State University
Baton Rouge

VIRGINIA R. WILLIAMS

Received for publication, April 13, 1945

ON THE EFFECT OF DRUGS ON CHOLINESTERASE

Sirs:

The concept that the release and removal of acetylcholine are responsible for the nerve action potential is based essentially on studies of the enzyme cholinesterase. By testing the rate of a number of choline and non-choline esters a pattern has been recently established which makes it possible to distinguish the enzyme specific for acetylcholine from other esterases.[1] All nerve tissues contain exclusively or predominantly the specific cholinesterase in high concentrations.

It appears of interest to test the affinity of certain drugs to cholinesterase for two reasons. First, to see whether it differs from that of other esterases. If this is the case, it would be further support for the assumption of the specific character of cholinesterase. Secondly, to find out whether compounds known to be stimulants of the central nervous system have an affinity to cholinesterase. This may possibly be relevant to their mode of action.

Compound	Cholinesterase				Unspecified esterase			
	Electric tissue		Ox brain		Horse serum		Guinea pig pancreas	
	Concentration $M \times 10^{-5}$	Inhibition	Concentration $M \times 10^{-5}$	Inhibition	Concentration $M \times 10^{-5}$	Inhibition	Concentration $M \times 10^{-5}$	Inhibition
		per cent		*per cent*		*per cent*		*per cent*
Caffeine	25	50	100	30	100	0	200	0
Quinine	100	50	100	33	2.5	51	5	33
Quinidine	50	50	100	22	1.25	61	2.5	35
Cocaine	100	25	200	27	12.5	51	12.5	52
Lobeline	12.5	30	50	22	1.56	50	3.12	44

The inhibitory effect of a variety of drugs has been studied on four enzyme preparations. Two of them represent specific cholinesterase, one prepared from electric tissue, the other from the nucleus caudatus of ox brain. The esterases from horse serum and from guinea pig pancreas were used as unspecified esterases. The first three preparations were purified;[2] the pancreas esterase was used without any purification.

Significant differences in affinity were found with a number of compounds

[1] Nachmansohn, D., and Rothenberg, M. A., *J. Biol. Chem.*, **158**, 653 (1945).

[2] Nachmansohn, D., and Rothenberg, M. A., in preparation.

shown in the table. As in the case of the specificity tests with choline and non-choline esters, the differences are mostly quantitative. Only caffeine and theobromine act exclusively on cholinesterase, an interesting fact in view of their well known action as general stimulants of the central nervous system. The data of the table may suffice as an illustration, but they do not give a complete picture, since the increase (or decrease) of the inhibitory effect frequently does not occur in proportion to the change in drug concentration. The weak effect of quinine on cholinesterase and its strong inhibition of other esterases may be of interest in connection with the strong antiesterase effect of atebrin.[3]

Other drugs tested were found to be equally weak inhibitors of both types of esterases, *e.g.* nicotine, or relatively strong inhibitors, *e.g.* strychnine and veratrine, acting in a concentration of the same order of magnitude on both types of esterase.

Department of Neurology
College of Physicians and Surgeons
Columbia University
New York

David Nachmansohn
Helen Schneemann

Received for publication, May 14, 1945

[3] Waelsch, H., and Nachmansohn, D., *Proc. Soc. Exp. Biol. and Med.*, **54**, 336 (1943).

COMPETITIVE INHIBITION OF PROCAINE CONVULSIONS BY ITS SPLIT-PRODUCTS

Sirs:

In experiments on the metabolic fate and mode of action of local anesthetics, we investigated the effect of the split-products of procaine upon the typical convulsive action of the drug in guinea pigs. There is evidence[1] to support the assumption that procaine is split in the body to *p*-aminobenzoic acid (PABA) and diethylaminoethanol (DEAE). It was found that 100 mg. per kilo of procaine hydrochloride, equal to 0.364 mM per kilo, given intramuscularly to guinea pigs produced convulsions in 80 per cent of the animals within 5 to 15 minutes. If, however, the procaine was given 30 minutes after an intraperitoneal injection of 400 mg. per kilo of PABA (as the sodium salt), equal to 2.9 mM per kilo, the convulsions occurred in only 15 per cent of the animals and were less intense. In analogous experiments, in which 400 mg. per kilo, equal to 3.42 mM per kilo, of DEAE (as the hydrochloride) were injected before the procaine, the incidence of convulsions was reduced to approximately 8 per cent and the severity of the seizures was markedly decreased. Smaller amounts of either PABA or DEAE afforded less or no protection against the convulsive action of procaine. Larger amounts of the split-products were necessary to protect the animals if the dose of procaine was raised.

Both DEAE and PABA are tolerated by the animals in large doses without eliciting noticeable symptoms. It was also found that these compounds do not exert an anticonvulsive action against metrazole. The present observations lend support to the assumption that the two split-products of procaine attach themselves to the same receptors on which procaine acts. This competition may take place directly on the cells of the central nervous system or may occur on some intermediary enzyme component such as cholinesterase. Studies devised to shed further light on this observation are in progress.

Department of Pharmacology
Abbott Laboratories
North Chicago

RICHARD K. RICHARDS

Received for publication, May 14, 1945

[1] Thieulin, J., *J. pharm. et chim.*, **22**, 463 (1920). Fosdick, L. S., and Hansen, H. L., *Dent. Cosmos*, **73**, 1082 (1931).

ISOCITRIC DEHYDROGENASE AND CARBON DIOXIDE FIXATION*

Sirs:

The biological breakdown of 1-isocitric acid to α-ketoglutaric acid and CO_2 has been assumed to take place through enzymatic dehydrogenation to oxalosuccinic acid, followed by spontaneous decarboxylation of the latter.[1] Adler *et al.*[2] demonstrated that triphosphopyridine nucleotide (TPN) and manganese ions are components of the system.

We have now shown that crude solutions of isocitric dehydrogenase contain two distinct enzymes that catalyze reversible Reactions 1 and 2.

$$(1)\quad \underset{\text{Isocitric acid}}{\mathrm{COOH\cdot CH_2\cdot \underset{\displaystyle COOH}{CH}\cdot CHOH\cdot COOH}} + \mathrm{TPN_{ox.}} \rightleftarrows \underset{\text{Oxalosuccinic acid}}{\mathrm{COOH\cdot CH_2\cdot \underset{\displaystyle COOH}{CH}\cdot CO\cdot COOH}} + \mathrm{TPN_{red.}}$$

$$(2)\quad \underset{\text{Oxalosuccinic acid}}{\mathrm{COOH\cdot CH_2\cdot \underset{\displaystyle COOH}{CH}\cdot CO\cdot COOH}} \rightleftarrows \underset{\alpha\text{-Ketoglutaric acid}}{\mathrm{COOH\cdot CH_2\cdot CH_2\cdot CO\cdot COOH}} + \mathrm{CO_2}$$

Reaction 1 is catalyzed by isocitric dehydrogenase and occurs in the absence of Mn^{++}. Reaction 2 is catalyzed by a specific enzyme, oxalosuccinic carboxylase,[3] and requires Mn^{++}. Summation of the two activities gives Reaction 3.

$$(3)\quad \mathrm{COOH\cdot CH_2\cdot \underset{\displaystyle COOH}{CH}\cdot CHOH\cdot COOH} + \mathrm{TPN_{ox.}} \rightleftarrows \mathrm{COOH\cdot CH_2\cdot CH_2\cdot CO\cdot COOH} + \mathrm{CO_2} + \mathrm{TPN_{red.}}$$

The enzyme solution used was a dialyzed extract of washed acetone-dried pig heart free from aconitase. Reactions 1 and 3 were followed by spectrophotometric measurement of $TPN_{red.}$ at 340 mμ.

* Supported by grants from the Rockefeller Foundation, the Penrose Fund of the American Philosophical Society, the Williams-Waterman Fund of the Research Corporation, and Hoffmann-La Roche, Inc.

[1] Martius, C., *Z. physiol. Chem.*, **247**, 104 (1937). However, Evans (*Harvey Lectures*, **39**, 285 (1943–44)) suggested that the decarboxylation might be enzymatic.

[2] Adler, E., von Euler, H., Günther, G., and Plass, M., *Biochem. J.*, **33**, 1028 (1939).

[3] Ochoa, S., and Weisz-Tabori, E., *J. Biol. Chem.*, **159**, 245 (1945).

In the absence of Mn^{++}, $TPN_{red.}$ (formed on mixing isocitric acid, heart enzyme, and TPN_{ox}) is reoxidized on addition of oxalosuccinic acid[4] (Reaction 1). In the presence of Mn^{++}, reoxidation can be brought about by α-ketoglutaric acid plus CO_2 (Reaction 3). If TPN is reduced with glucose-6-phosphate and *Zwischenferment*,[5] reoxidation is caused by α-ketoglutaric acid plus CO_2 only after adding heart enzyme and in the presence of Mn^{++}.

The equilibrium constant of Reaction 1, K_1 = (l-isocitrate)($TPN_{ox.}$)/(oxalosuccinate)($TPN_{red.}$), is about 0.3 at pH 7 and 22°. That of Reaction 3, K = (l-isocitrate)($TPN_{ox.}$)/(α-ketoglutarate)(CO_2)($TPN_{red.}$) at widely different concentrations of reactants (CO_2 in the atmosphere in equilibrium with the reaction mixtures from 2.8 to 47 per cent), has an average value of 1.3×10^{-4} at pH 7 and 22°. Hence the equilibrium constant of the oxalosuccinic carboxylase (Reaction 2) is K_2 = (oxalosuccinate)/(α-ketoglutarate)(CO_2) = $K/K_1 = 1.4 \times 10^{-4}/0.3 = 0.5 \times 10^{-3}$.

The equilibrium of Reaction 3 can be shifted to the left, *i.e.* toward CO_2 fixation, by combination with the glucose-6-phosphate dehydrogenase system. In this case $TPN_{ox.}$ is reduced according to the reaction, glucose-6-phosphate + $TPN_{ox.}$ → 6-phosphogluconate + $TPN_{red.}$. The net result is the dismutation, α-ketoglutarate + CO_2 + glucose-6-phosphate = l-isocitrate + 6-phosphogluconate. The isocitrate formed in this dismutation was determined with isocitric dehydrogenase.

Further displacement of equilibrium, favoring carboxylation of α-ketoglutaric acid, would occur in the presence of aconitase, since over 90 per cent of the isocitric acid would be removed to form *cis*-aconitate and citrate.[6] Thus the aconitase-isocitric dehydrogenase-oxalosuccinic carboxylase system might play an important part in the biological utilization of CO_2.

Departments of Chemistry and Medicine
New York University College of Medicine
New York

SEVERO OCHOA

Received for publication, May 16, 1945

[4] Oxalosuccinic acid has been prepared for the first time by cold acid hydrolysis of its triethyl ester and isolated as the barium salt. The ester was prepared by the method of W. Wislicenus and M. Waldmüller (*Ber. chem. Ges.*, **44**, 1564 (1911)).

[5] Kindly supplied by Dr. Erwin Haas.

[6] Martius, C., and Leonhardt, H., *Z. physiol. Chem.*, **278**, 208 (1943).

OXALOSUCCINIC CARBOXYLASE*

Sirs:

As stated in the foregoing Letter[1] crude solutions of isocitric dehydrogenase from pig heart contain, in addition to the dehydrogenase, an enzyme that catalyses the reversible reaction

$$\underset{\text{Oxalosuccinic acid}}{\mathrm{COOH{\cdot}CH_2{\cdot}\underset{\displaystyle COOH}{CH}{\cdot}CO{\cdot}COOH}} \rightleftarrows \underset{\alpha\text{-Ketoglutaric acid}}{\mathrm{COOH{\cdot}CH_2{\cdot}CH_2{\cdot}CO{\cdot}COOH}} + \mathrm{CO_2}$$

To the right, *i.e.* decarboxylation of oxalosuccinic acid, the reaction can be followed manometrically, and, since oxalosuccinic acid is relatively unstable in aqueous solution, it is best to study the enzymatic breakdown at low temperature, *viz.* 12–14°.

Enzymatic Decarboxylation of Oxalosuccinic Acid

0.018 M citrate buffer, pH 5.6, and 19 micromoles of either oxalosuccinic or oxaloacetic acid (equivalent to 425 c.mm. of CO_2) in a final volume of 2.8 cc. Enzyme, 1.0 cc. of dialyzed extract of washed, acetone-dried, pig heart containing about 5 mg. of protein. Substrates tipped in from the side bulb of the Warburg vessels after temperature equilibration. Gas, air.

Additions	CO_2 evolved in 15 min.	
	Oxalosuccinate (14°)	Oxaloacetate (25°)
	c.mm.	*c.mm.*
None	54	37
0.0013 M $MnCl_2$	76	38
Enzyme	67	37
" + 0.0013 M $MnCl_2$	314	42
Heated enzyme (2 min. at 100°) + 0.0013 M $MnCl_2$	62	

The enzyme is thermolabile and acts only in the presence of manganese ions. Since the heart extracts do not catalyze the decarboxylation of oxaloacetic acid, oxalosuccinic carboxylase is a specific enzyme.

In comparison with manganese at the same molar concentration, magnesium has almost no effect on the enzymatic decarboxylation of oxalosuccinic

* Supported by grants from the Rockefeller Foundation, the Penrose Fund of the American Philosophical Society, the Williams-Waterman Fund of the Research Corporation, and Hoffmann-La Roche, Inc.

[1] Ochoa, S., *J. Biol. Chem.*, **159**, 243 (1945).

acid. Similar results were obtained by Evans *et al.*[2] with the oxaloacetic carboxylase from pigeon liver.

The rate of enzymatic decarboxylation of oxalosuccinic acid is proportional to the enzyme concentration. The carboxylase can be precipitated from aqueous solutions with acetone without loss of activity, and is almost completely precipitated by acidification to pH 5.2, which must therefore be close to the isoelectric point of the enzyme.

The spontaneous (non-enzymatic) decarboxylation of oxalosuccinic acid has been studied under various conditions in comparison with that of oxaloacetic acid. At 25° and pH 5.1 (acetate buffer) the first order velocity constant of decarboxylation of oxalosuccinic acid was $k_{OSA} = 1.4 \times 10^{-2}$, that of oxaloacetic decarboxylation $k_{OAA} = 1.5 \times 10^{-3}$.

As reported by Krebs[3] for oxaloacetic acid, we found that polyvalent cations such as Cu^{++}, Fe^{++}, Fe^{+++}, and Al^{+++} (1×10^{-3} M) greatly accelerate the spontaneous decarboxylation of oxalosuccinic acid. At the same concentration, however, Ca^{++}, Mg^{++}, and Mn^{++} have very little effect. The decarboxylation of oxalosuccinic acid is also markedly accelerated by aniline.

Departments of Chemistry and Medicine
New York University College of Medicine
New York

SEVERO OCHOA
ERNA WEISZ-TABORI

Received for publication, May 16, 1945

[2] Evans, E. A., Jr., Vennesland, B., and Slotin, L., *J. Biol. Chem.*, **147**, 771 (1943).
[3] Krebs, H. A., *Biochem. J.*, **36**, 303 (1942).

A DIETARY FACTOR ESSENTIAL FOR GUINEA PIGS

VII. CHANGES IN THE DISTRIBUTION OF THE PLASMA PROTEIN DURING THE DEFICIENCY*

By WILLEM J. VAN WAGTENDONK AND LEONARD P. ZILL†

(*From the Department of Chemistry, Oregon State College, Corvallis*)

(Received for publication, March 26, 1945)

The concentration of serum protein in normal humans is comparatively constant, ranging from 6.0 to 8.0 gm. per 100 cc. (1–3). The range of albumin is from 3.6 to 5.4 gm. per 100 cc. and that of globulin from 1.5 to 3.4 gm. per 100 cc. From the values reported in the literature the range for the albumin-globulin ratio may be given as 1.2 to 2.6. The estimation of the serum proteins in the various forms of renal disease is of considerable value. In non-hemorrhagic degenerative Bright's disease or nephrosis the serum protein is decreased markedly and this is due almost entirely to a deficit of albumin (1, 4). The tendency toward edema in nephrosis is closely related to the serum albumin concentration. In acute glomerular nephritis the plasma protein concentration may remain within normal limits, although in severe cases considerable deficits may be encountered (5). Lowering of the serum protein level, which is chiefly at the expense of the albumin fraction, is also found in cirrhosis of the liver (6, 7). Non-protein nitrogen retention is experienced not only in impairment of renal function or urinary obstruction but is encountered also in instances of excessive tissue protein catabolism.

There is also a close relationship between the calcium and protein content of the serum. McLean and Hastings (8) have demonstrated that total serum calcium, calcium ions, and total serum proteins are related in a manner which can be stated in an equilibrium equation. Their experiments led to the conclusion that the calcium of the serum is present almost entirely as calcium ions and calcium bound to protein. In cases of an abnormality in the calcium concentration in blood serum the possibility exists that it is associated with an abnormal distribution of the serum proteins.

* Supported by grants from the Williams-Waterman Fund of the Research Corporation and from the General Research Council of the Oregon State System of Higher Education.

† Based on data from a thesis submitted by one of the authors (L. P. Z.) as partial fulfilment of the requirements for the degree of Master of Science, Oregon State College. A part of this study was presented before the Division of Biological Chemistry at the 108th meeting of the American Chemical Society at New York, September 11–15, 1944.

It has been found in previous investigations that a deficiency of the antistiffness factor results, among other pathological manifestations (9), in an abnormal distribution of the acid-soluble phosphorus in the liver and kidneys of the experimental animals, probably associated with damaged hepatic and renal function (10), an increase in the level of serum phosphate and serum calcium (11), and a lower level of serum phosphatase (12). An abnormal distribution of the serum proteins could be expected as a result of or in conjunction with the above mentioned changes in phosphorus and calcium metabolism and might explain several of the symptoms of the deficiency. The present investigation was undertaken with this purpose in mind.

EXPERIMENTAL

A group of 250 guinea pigs was used for this experiment. The animals were raised on the deficient diet as described by van Wagtendonk (10). The diet consisted of skim milk powder to which adequate amounts of minerals and the known vitamins had been added. The animals were sacrificed at various intervals. The blood was collected by cardiac puncture under nembutal anesthesia and was sampled in tubes containing heparin (2 mg. per 15 cc. of blood) to prevent clotting. After centrifuging for 10 minutes at 4000 R.P.M., the supernatant serum was used for the determination of the distribution of the proteins according to the procedure of Greenberg (13). For the precipitation of globulin 0.25 cc. of plasma was diluted to 8 cc. with 22.5 per cent Na_2SO_4 solution. After a small crystal of thymol was added, the solution was incubated for 5 hours at 37°. The solution was then filtered (Whatman, No. 42). The filtrate contains the albumin and non-protein nitrogenous compounds. The precipitate, representing the globulin fraction, was dissolved in dilute NaOH solution. For the determination of the total protein and non-protein fractions 0.75 cc. of plasma was diluted to 7.5 cc. with isotonic NaCl solution. 2 cc. of this solution were used for the total nitrogen determination. The remainder of the solution was treated with 5.5 cc. of 10 per cent trichloroacetic acid and filtered after 10 minutes. A 5 cc. sample was used for the determination of the non-protein nitrogenous compounds. The nitrogen content of the fractions so obtained was determined by the micro-Kjeldahl method, with the digestion mixture recommended by Rinehart *et al.* (14). All fractions were calculated as nitrogen. The data were analyzed statistically according to the methods of Fisher (15).

Results

In Table I are presented the mean values of the different nitrogen fractions in the plasma of guinea pigs receiving "normal" diets as follows:

(*a*) the stock diet composed of rolled barley, greens, iodized salt, straw, and water *ad libitum*, and (*b*) a raw milk diet for 70 weeks. As was expected, the distribution of the plasma proteins remained essentially unchanged during the period investigated. In those animals receiving the raw milk diet a slightly (but not significantly) higher concentration of all the fractions with the exception of the globulin fraction was found. The

TABLE I

Distribution of Nitrogen in Plasma of Guinea Pigs Receiving Stock Diet

Age	No. of determinations	Mean and standard error, mg. per 100 cc.					Albumin / Globulin
		Total N	Total protein N	Non-protein N	Albumin N	Globulin N	
wks.							
10	5	719 ± 17	691 ± 16	28 ± 3	357 ± 36	334 ± 34	1.07
12	5	684 ± 13	663 ± 15	21 ± 3	308 ± 42	356 ± 52	0.87
16	15	749 ± 14	721 ± 16	28 ± 2	342 ± 20	380 ± 24	0.95
72	10	707 ± 52	639 ± 53	68 ± 4	319 ± 31	320 ± 32	1.00
72*	10	842 ± 69	759 ± 67	83 ± 5	410 ± 91	348 ± 37	1.18

* This group of animals had received a raw milk diet for 70 weeks.

TABLE II

Distribution of Nitrogen in Plasma of Guinea Pigs Receiving Diet Deficient in Anti-stiffness Factor

Age	Time on diet	No. of determinations	Mean and standard error, mg. per 100 cc.					Albumin / Globulin
			Total N	Total protein N	Non-protein N	Albumin N	Globulin N	
wks.	*wks.*							
14	1	15	749 ± 23	684 ± 27	56 ± 4	228 ± 27	466 ± 31	0.49
15	2	15	844 ± 16	781 ± 19	58 ± 3	345 ± 16	441 ± 26	0.65
16	3	15	795 ± 15	750 ± 17	45 ± 4	204 ± 20	546 ± 32	0.37
20	7	8	911 ± 26	827 ± 26	84 ± 13	215 ± 31	612 ± 38	0.35
41	28	15	923 ± 22	855 ± 23	67 ± 3	224 ± 42	631 ± 48	0.36
70	57	16	890 ± 26	816 ± 29	82 ± 3	298 ± 37	533 ± 45	0.56

albumin-globulin ratio was higher in these animals than in those on the stock diet.

Values for the protein distribution in animals raised on a deficient diet are given in Table II. The total protein nitrogen showed a significant increase during the deficiency. This was mainly due to an increase in the globulin and non-protein nitrogen fraction. The albumin nitrogen was significantly lower in the deficient animals than in the normal ones. As a consequence the albumin-globulin ratio was much lower in the de-

ficient animals than in the normal ones, a difference which was already evident in the 1st week after the animals had been started on the skim milk diet.

It was shown previously that an abnormal distribution of the acid-soluble P in the liver and kidneys of deficient animals could be restored to normal by the administration of the antistiffness factor in five con-

TABLE III

Effect of "Cure" with Antistiffness Factor on Distribution of Plasma Nitrogen of Guinea Pigs Raised on Skim Milk Diet

Experiment No.	Age	Time on diet	Deficient: No. of determinations	Deficient: Mean ± standard error, mg. per 100 cc.: Total N	Total protein N	Non-protein N	Albumin N	Globulin N	Deficient: Albumin/Globulin	Supplement of antistiffness factor: Dosage of antistiffness factor	No. of determinations	Mean ± standard error, mg per 100 cc.: Total N	Total protein N	Non-protein N	Albumin N	Globulin N	Albumin/Globulin
	wks.	*wks.*								γ							
1	20	7	8	911 ±26	827 ±26	84 ±13	215 ±31	612 ±38	0.35	0.5*	7	910 ±11	823 ±47	86 ±9	224 ±49	599 ±45	0.37
2	32	19	5	870 ±18	779 ±20	91 ±8	213 ±69	567 ±74	0.37	0.5*	10	920 ±22	841 ±22	79 ±1	296 ±40	544 ±31	0.54
	32	19								5†	5	901 ±33	821 ±34	80 ±3	337 ±35	480 ±85	0.70
3	81	68	8	990 ±20	884 ±21	106 ±10	204 ±30	680 ±45	0.30	50‡	10	800 ±26	718 ±20	80 ±2	360 ±25	358 ±20	1.00
	81	68								100§	8	840 ±20	758 ±20	82 ±1	410 ±30	341 ±20	1.18
4	23	10								1‖	14	887 ±11	816 ±11	71 ±3	402 ±21	413 ±19	0.97

* Administered in daily dosages of 0.1 γ during the last 5 days of the experiment.
† Administered in daily dosages of 1 γ during the last 5 days of the experiment.
‡ Administered in daily dosages of 1 γ during the last 50 days of the experiment.
§ Administered in daily dosages of 1 γ during the last 100 days of the experiment.
‖ Administered every other day during the experiment.

secutive dosages of 100 to 1000 units (0.1 to 1 γ). It can be seen from Experiments 1 and 2 reported in Table III that there is no speedy recovery in the abnormal protein distribution following the administration of this factor. The influence of the antistiffness factor in the dosages employed here is slight, although some improvement can be noted. If, however, the antistiffness factor is given for a longer period (Experiment 3 of Table III), a return to normal condition results. An abnormal distribution of the

serum proteins can be prevented by a continuous administration of the factor, as is clearly demonstrated in Experiment 4.

DISCUSSION

Abnormalities in the protein distribution in the plasma are important if considered in conjunction with abnormal values for other blood constituents, in particular the changes which may occur in the plasma calcium and plasma phosphorus. In a preceding paper of this series (11) high values for the plasma calcium and the plasma phosphorus during the deficiency of the antistiffness factor were reported. It was now found that this high calcium and phosphorus content was accompanied by a low albumin-globulin ratio. The albumin concentration was lowered far below normal in deficient animals.

In contrast to the immediate restoration of the easily hydrolyzable P fraction in the liver to the original level after administration of the antistiffness factor to deficient animals the protein distribution is normalized only after a prolonged treatment. A healthy condition in the liver is a prerequisite for the direction of the protein synthesis into normal channels. That this condition is not immediately attained after administration of the factor is evident from the experiments reported here and is in accordance with the findings reported earlier (10). For although the fraction of easily hydrolyzable P returned to normal after administration of the factor, a high concentration of inorganic P was still evident. The abnormal distribution of the serum proteins is therefore probably a secondary symptom of the deficiency. Owing to the effect of the deficiency on the phosphorus metabolism in the liver, the hepatic function is damaged, resulting in deranged protein synthesis.

About 62 per cent of the total calcium found in the plasma is present as diffusible calcium (16). Evidence points to the fact that the serum albumin rather than the serum globulin contributes most largely to the binding of calcium. Gunther and Greenberg (17) noted that the albumin and calcium content decreased simultaneously during jaundice and neoplastic diseases. Csapo and Faubl (18) and Bendien and Snapper (19) came to the conclusion that the non-diffusible calcium is completely united with the albumin. The level of the serum protein concentration contributes toward the height of the serum calcium. A high level of the serum protein is usually associated with an increased level of serum calcium. A decrease of the amount of serum calcium in cases of Bright's disease parallels the reduction in the content of the serum proteins.

In the animal deficient in the antistiffness factor a somewhat higher calcium level is associated with a high protein level. However, the albumin concentration is greatly decreased during the deficiency disease. The

amount of calcium bound to the albumin must, therefore, have decreased accordingly. Since the calcium concentration is higher than in the normal animal, a higher concentration of the unbound calcium must be present in the blood. Also a significant increase of inorganic phosphate takes place during the deficiency. The conditions for the formation of a colloidal form of calcium phosphate are therefore favorable, as was demonstrated by Scholtz (20), Laskowski (21), and Greenberg *et al.* (22).

It is now possible to integrate to some extent the disturbances caused by a deficiency of the antistiffness factor. Apparently an early symptom of the deficiency is the rapid decrease in easily hydrolyzable phosphorus in the liver and kidneys. As a result of this the mercuric-insoluble phosphorus and the inorganic phosphate increase. Some of the excess of the inorganic phosphate is excreted in the urine. Owing to this change in P metabolism, the normal hepatic function is disturbed, resulting in a deranged protein synthesis. In the muscle the adenosine triphosphate and the creatine phosphate concentrations are lowered, while the inorganic phosphate is increased (23). The alkaline serum phosphatase activity is also lower during the deficiency. While the total calcium concentration does not increase materially, the concentration of the unbound calcium increases owing to a lower level of the albumin fraction. As a result of the higher concentrations of calcium and inorganic phosphate in the blood and tissues, colloidal calcium phosphate is formed which may precipitate whenever the conditions are favorable for such a reaction.

It is realized that no satisfactory answer has been given as to the mode of action of this compound (enzyme or coenzyme?). These questions cannot be answered until the antistiffness factor has been identified. Work along this line is in progress.

SUMMARY

Significant changes in the plasma protein distribution take place during a deficiency of the antistiffness factor. The total plasma nitrogen shows a significant increase due mainly to the rise in globulin nitrogen content. The albumin nitrogen concentration on the other hand decreases. As a result the albumin-globulin ratio is much lower in the plasma of deficient guinea pigs than in that of normal animals. A return to normal values is evident only after a prolonged administration of the antistiffness factor. The implications of these changes are discussed.

BIBLIOGRAPHY

1. Fahr, G., and Swanson, W. W., *Arch. Int. Med.*, **38**, 510 (1926).
2. Moore, N. S., and Van Slyke, D. D., *J. Clin. Invest.*, **8**, 337 (1930).
3. Muntwyler, E., Way, C. T., Binns, D., and Myers, V. C., *J. Clin. Invest.*, **12**, 495 (1933).

4. Van Slyke, D. D., McIntosh, J. F., Möller, E., Hannon, R. R., and Johnston, C., *J. Clin. Invest.*, **8**, 357 (1930).
5. Peters, J. P., Bruckman, F. S., Eisenman, A. J., Hald, P. N., and Wakeman, A. M., *J. Clin. Invest.*, **11**, 97 (1932).
6. Butt, H. R., Snell, A. M., and Keys, A., *Arch. Int. Med.*, **63**, 143 (1939).
7. Post, J., and Patek, A. J., Jr., *Bull. New York Acad. Med.*, **19**, 815 (1943).
8. McLean, F. C., and Hastings, A. B., *J. Biol. Chem.*, **108**, 285 (1935).
9. van Wagtendonk, W. J., and Wulzen, R., *Arch. Biochem.*, **1**, 373 (1943).
10. van Wagtendonk, W. J., *J. Biol. Chem.*, **155**, 337 (1944).
11. van Wagtendonk, W. J., Freed, A. M., and Ballou, C. E., *Arch. Biochem.*, **5**, 329 (1944).
12. van Wagtendonk, W. J., Rathkey, A. S., Ballou, C. E., and Wulzen, R., *Arch. Biochem.*, **5**, 273 (1944).
13. Greenberg, D. M., *J. Biol. Chem.*, **82**, 545 (1929).
14. Rinehart, R. E., Grondahl, R. D., and West, E. S., *Arch. Biochem.*, **2**, 163 (1943).
15. Fisher, R. A., Statistical methods for research workers, Edinburgh, 8th edition (1941).
16. Greene, C. H., and Powers, M. H., *J. Biol. Chem.*, **91**, 183 (1931).
17. Gunther, L., and Greenberg, D. M., *Arch. Int. Med.*, **45**, 67, 983 (1930); **47**, 660 (1931).
18. Csapo, J., and Faubl, J., *Biochem. Z.*, **150**, 509 (1924).
19. Bendien, W. M., and Snapper, I., *Biochem. Z.*, **260**, 105 (1933).
20. Scholtz, H. G., *Biochem. Z.*, **249**, 394 (1933).
21. Laskowski, M., *Biochem. Z.*, **265**, 401 (1933).
22. Greenberg, D. M., Lucia, S. P., Mackey, M. A., and Tufts, E. V., *J. Biol. Chem.*, **100**, 139 (1933).
23. van Wagtendonk, W. J., and Lamfrom, H., *J. Biol. Chem.*, **158**, 421 (1945).

A MONOLAYER AND X-RAY STUDY OF MYCOLIC ACID FROM THE HUMAN TUBERCLE BACILLUS

By STINA STÄLLBERG-STENHAGEN and EINAR STENHAGEN

(*From the Institute of Medical Chemistry and the Institute of Physiology, University of Upsala, Upsala, Sweden*)

(Received for publication, January 29, 1945)

The mycolic acids are the principal ether-soluble constituents of the waxes of acid-fast bacteria and have been isolated and studied by Anderson and his collaborators (1–5). Chemically the mycolic acids have been found to be hydroxy or hydroxymethoxy acids of very high molecular weight. Compounds of the same general type but of slightly different composition have been found in different acid-fast bacteria. The mycolic acids are acid-fast; *i.e.*, they are responsible for the characteristic staining properties of this group of bacteria (1). The determination of the structure of these compounds is a very important but difficult matter and the work in this direction with ordinary chemical methods has as yet not proceeded very far. Professor Anderson has been kind enough to place a number of mycolic acids at our disposal for monolayer and x-ray studies.

Mycolic acid (the compound isolated from the human tubercle bacillus carries this name only; those from other acid-fast bacteria are denoted by suitable prefixes) has according to Stodola, Lesuk, and Anderson (1) the composition $C_{88}H_{172}O_4$ or $C_{88}H_{176}O_4$. The oxygen atoms are present as one carboxyl, one hydroxyl, and one methoxyl group. Mycolic acid melts at 54–56° and the specific rotation (in chloroform) is $[\alpha]_D^{25} = +1.8°$. The compound has the appearance of a white amorphous powder. On heating under reduced pressure at 300–350°, *n*-hexacosanoic acid distils off. Lesuk and Anderson (5) prepared normycolic acid by reduction of diiodonormycolic acid and were able to isolate from the reaction product also a small amount of a monohydroxymonocarboxylic acid with a higher molecular weight than the original mycolic acid. The acid had the approximate composition of $C_{104}H_{208}O_3$ and gave *n*-hexacosanoic acid on pyrolysis. Lesuk and Anderson concluded that the original mycolic acid probably contained two acids with very similar solubility properties. The relative proportion of the two components is as yet unknown; *i.e.*, it is not certain that the C_{104} acid can be regarded as a minor impurity. On oxidation with chromic acid mycolic acid gave stearic acid, *n*-hexacosanoic acid, and 1,16-hexadecanedicarboxylic acid.

EXPERIMENTAL

Monolayer Investigation—The technique has been described previously (6). The melting point of the specimen of mycolic acid used was given

as 55–56°. The oxygen content of the sample was checked by a microanalytical determination of oxygen by the Schütze-Unterzaucher method (7).

Analysis—$C_{88}H_{176}O_4$ (1296). Calculated, O 4.93; found, O 4.86.

Spreading was effected from a solution in petroleum ether (b.p. 60–80°). The area values have been calculated with the formula $C_{88}H_{176}O_4$.

The force-area curves for mycolic acid in the undissociated state at different temperatures are shown in Fig. 1. At temperatures below 35°

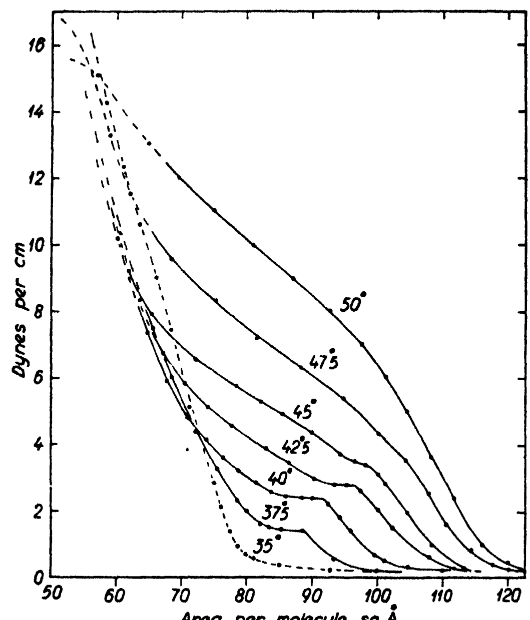


Fig. 1. Force-area curves for mycolic acid monolayers at different temperatures when spread on 0.01 N hydrochloric acid.

mycolic acid forms a plastic solid film which is very unstable even at the lowest surface pressures. Above this temperature expansion takes place and the expanded monolayer is more stable. At 40°, the limiting area is 106 sq. A and transition into the condensed state starts at an area of 90 sq. A and a pressure of 2.5 dynes. The monolayer is reasonably stable up to about 7 dynes pressure, but above this point a gradual collapse sets in. In the expanded region below 2.5 dynes pressure the monolayer is liquid but rather viscous and begins to show elastic properties somewhat below the transition point. Although the monolayer very much resembles the liquid-expanded type (8), it is doubtful whether it is a true liquid even at the limiting area. After the transition the monolayer gradually solid-

ifies to an elastic solid. On reexpansion from 10 dynes pressure the force-area curve falls below that obtained on the original compression, indicating that an irreversible partial collapse has taken place. The limiting area and the lower part of the force-area curve are reproducible within less than 2 per cent in different runs. At 50° the monolayer is fully expanded. The temperature of half expansion under 1.5 dynes pressure is about 41°.

The results obtained with the Langmuir-Adam balance were checked by a series of experiments with a recording surface balance of the Wilhelmy-Dervichian type (9).

The behavior of mycolic acid monolayers on different substrates at a temperature of 45° has been studied. The force-area curve is only very slightly changed by a change in the hydrogen ion concentration of the substrate, but there is a small increase in the limiting area on 0.01 N sodium hydroxide. Barium ions in a neutral substrate ($BaCl_2$, 3×10^{-4} M; $KHCO_3$, 4×10^{-3} M) have a slight condensing effect.

Although at 20° the monolayer is very unstable and the pressure after a compression rapidly falls nearly to zero, it was found that the surface potential first rises and then, on further reduction of the area, attains a constant value. This "saturation" potential is usually constant within 10 millivolts for different parts of the film, and the values found in different runs usually agreed within 15 millivolts, although sometimes larger deviations were found. The saturation potential for mycolic acid at different pH values is shown in Fig. 2. The substrates having a pH of 2 or lower consisted of hydrochloric acid in appropriate concentration, from pH 3 to 11 they consisted of a universal buffer (10), diluted 10 times, and the substrate of pH 12 was made up of 0.01 N sodium hydroxide. The values for the normal chain myristic acid given by Schulman and Hughes (11), who employed a similar procedure of measuring the potential of a surface excess of the substance, are also included in Fig. 2 for comparison.

The surface potential of undissociated mycolic acid is about the same, 400 millivolts, as that of the normal chain myristic acid. The rise in potential observed for normal chain acids on very strong acid (4 N hydrochloric acid) is probably due to oxonium complex formation (11). The three polar groups of mycolic acid are all capable of oxonium complex formation, but in spite of this we found no rise in the surface potential in this region. At pH values higher than 4 the surface potential falls as a result of ionization. Above pH 10 the acid is fully ionized and the surface potential remains fairly constant at about 100 millivolts. As the surface potential of hydroxyl and methoxyl groups is constant between pH 2 and 10 (11), the change in potential is entirely due to ionization of the carboxyl group, provided that there is no change in potential in this region due to reorientation of the hydroxyl or methoxyl groups. As the type of mono-

layer remains apparently the same, reorientation is not likely and the pH value at the point of inflection of the surface potential-pH curve thus probably corresponds to the point of half dissociation of mycolic acid, *i.e.*, to the (monolayer) dissociation constant. Mycolic acid with a pK (monolayer) of about 7.5 thus appears to be an acid of about the same strength as the normal chain myristic acid.

Attempts to build multilayers of mycolic acid with various substrates and temperatures were unsuccessful.

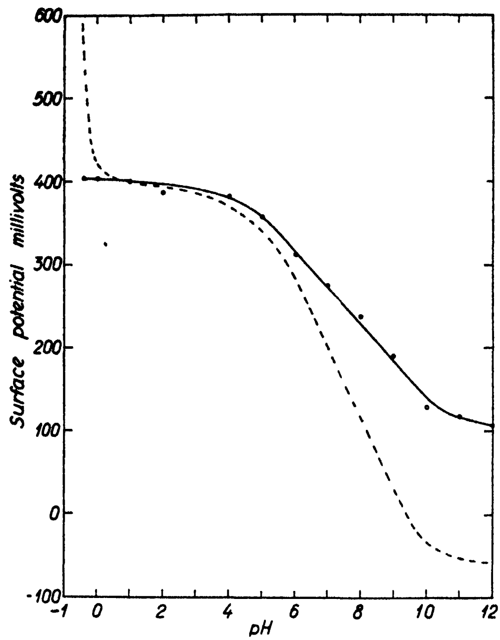


FIG. 2. Variation of "saturation potential" with pH of substrate for mycolic acid monolayers at 20°. The values for the normal chain myristic acid given by Schulman and Hughes (11) are shown in the dash line curve.

It is remarkable that the large mycolic acid molecule which contains only 4 oxygen atoms, distributed within three different polar groups, for 88 carbon atoms, forms a monolayer of the liquid-expanded type. Liquid-expanded monolayers showing sharp transition points have as yet been found among branched chain compounds only if the molecule has a long chain with comparatively short branches. It is evident that the carboxyl group anchors the molecule to the surface but it appears very unlikely that the carboxyl group alone would be sufficient to cause spreading. The structure of the molecule therefore probably allows access to the water of at least one of the other polar groups. The monolayer experiments also

show that the cross-section of the molecule at no part of its length can be larger than that occupied by three parallel hydrocarbon chains.

x-Ray Investigation—Specimens of mycolic acid suitable for x-ray investigation were prepared by carefully pressing a small amount of the acid onto a small glass slide (6 × 20 mm.) or melting the acid onto glass by means of the hot wire technique (12). The x-ray investigation was carried out in the usual manner with nickel-filtered copper K_α radiation (13). The distance from specimen to photographic film was 10.0 cm.

The diffraction pattern of a pressed specimen of the acid (as received or reprecipitated from solvents) showed three side spacings of 4.58, 3.86, and 3.57 A respectively. The 4.58 A spacing was strong and fairly sharp, the two others weaker (3.57 A weakest) and somewhat diffuse. In spite of very long exposures no trace of a long spacing was observed. It is

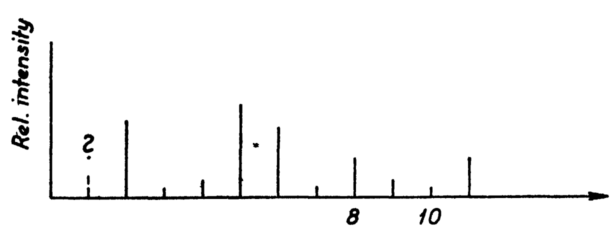


FIG. 3. Approximate relative intensities of the 00l reflections of the α form of mycolic acid. Intensity of the first order was difficult to estimate owing to the strong background around the central spot resulting from the long exposures needed.

evident that, probably also due to the inhomogeneity of the acid, the tendency to crystallize is poor. A specimen that had been melted onto glass showed only one very broad and diffuse side spacing of about 4.2 A, but long exposure (more than 20 hours with a Philips type 25 101 tube running at 30 kilovolts and 20 milliamperes) also brought out a series of orders of a very long spacing of 100 ± 1.5 A. The estimated relative intensities of the latter are recorded in Fig. 3. These might be useful for checking any proposed structure for mycolic acid. At present too little is known about the crystalline arrangement and the structure of the molecule to make any calculations profitable.

These results suggest that the acid exists in two crystalline modifications. The behavior of mycolic acid on melting supports this conclusion. On heating a specimen of the acid (obtained by precipitation from solvents) in a capillary tube at a rate of 1° every 5 minutes, a slight sintering starts

at 52–53°. Above 55° the material becomes increasingly transparent and it is fully transparent at about 56.5°, but does not liquefy completely until a temperature of about 58° is reached. On cooling the melt, the exact point of solidification is difficult to determine, but the material is still fully transparent at 53°. On further cooling the material becomes translucent rather than transparent and retains this appearance on being kept at room temperature.

The thermal behavior of mycolic acid may be compared with that of long normal chain hydrocarbons. At lower temperature these exist in opaque crystalline forms with vertical or tilted, non-rotating chains (14, 15). At a temperature a few degrees below the melting point a transition occurs to a transparent form. The series of side spacings given by the opaque form disappears in favor of a single side spacing of 4.1 to 4.2 A, but for the opaque form in which the chains are vertical (normal or A form) there is no appreciable change in the long spacing. In the transparent form thus obtained the chains are vertical and rotating and as a result the packing of the chains shows a higher (hexagonal) symmetry (14). The single side spacing of about 4.2 A is typical for rotating or in the solid state randomly distributed hydrocarbon chains (smectic state (16)). The transparent α forms of ethyl esters and glycerides belong to this class. In these cases the chains are always vertical.

Applied to mycolic acid the above would suggest the following. Mycolic acid obtained from solvents crystallizes in a non-rotating, opaque form. The crystallites are probably very small and it cannot be decided whether the chains are vertical or tilted. On heating above 52°, there is a gradual change to a transparent form with vertical rotating chains which is fully developed before final melting sets in. On cooling the acid first solidifies in this transparent α form. On further cooling below 53° the material becomes translucent rather than transparent and it is probable that at lower temperatures the chains are no longer rotating but that the high viscosity of the material prevents the large and awkwardly shaped molecules from taking up a more orderly arrangement and the molecules are therefore simply "frozen" with vertical chains arranged in a random fashion. In view of the fact that mycolic acid is probably inhomogeneous it might be pointed out that the presence of homologous impurities tends to stabilize otherwise metastable α forms (15). The long spacing of 100 A given by melted specimens of mycolic acid therefore most probably corresponds to the length of one or two mycolic acid molecules, depending on whether the arrangement is one with single or double molecules respectively. As layers with vertically oriented rotating or randomly distributed molecules would behave optically as a uniaxial crystal, specimens prepared by the technique described by Bernal and Crowfoot (12) were examined in polarized

light. It was found impossible to prepare specimens that were sufficiently well oriented, however, and no definite conclusion could be drawn from the optical examination. It appears safe, however, to conclude that the length of the mycolic acid molecule is either 50 or 100 A. Carboxylic acids have previously always been found to crystallize with a double molecule arrangement. Mycolic acid possesses 87 carbon atoms in the hydrocarbon chain. An unbranched hydrocarbon chain containing this number of carbon atoms would have a length of 111 A and a length of the molecule in the crystal of only 50 A would require some form of "hairpin" molecule, somewhat resembling that of the diglycerides. Opposed to this, as has been pointed out above, is the fact that up to now monolayers of the liquid-expanded type showing sharp transitions have only been given by branched chain molecules that consist of a long chain with only short side chains.

It might be possible to settle this question by an x-ray investigation of suitable derivatives of mycolic acid.

We are greatly indebted to Professor R. J. Anderson for providing us with a sample of mycolic acid and for his interest in the work. Financial support from the National Swedish Antituberculosis Association and the Rockefeller Foundation is gratefully acknowledged.

SUMMARY

Mycolic acid gives monolayers resembling the liquid-expanded type with a temperature of half expansion of 41°. The monolayer is unstable when the area per molecule is reduced below 60 sq. A. Mycolic acid is microcrystalline and specimens precipitated from solvents give x-ray diffraction patterns showing three side spacings of 4.58, 3.86, and 3.57 A respectively. Melted specimens show one very diffuse side spacing of 4.2 A and also several orders of a long spacing of 100 A. The behavior of the substance indicates that in the latter modification the molecules are vertical. The length of the molecule is therefore probably either 50 or 100 A, depending on whether the arrangement is one with single or double molecules. Further work on suitable derivatives is necessary to settle this question. Possibly due to inhomogeneity of the acid the tendency to crystallize is poor and no long x-ray spacing has been obtained from specimens prepared from solvents.

BIBLIOGRAPHY

1. Stodola, F. H., Lesuk, A., and Anderson, R. J., *J. Biol. Chem.*, **126**, 505 (1938).
2. Cason, J., and Anderson, R. J., *J. Biol. Chem.*, **126**, 527 (1938).
3. Anderson, R. J., and Creighton, M. M., *J. Biol. Chem.*, **129**, 57 (1939).
4. Anderson, R. J., Creighton, M. M., and Peck, R. L., *J. Biol. Chem.*, **133**, 675 (1940).
5. Lesuk, A., and Anderson, R. J., *J. Biol. Chem.*, **136**, 603 (1940).

6. Ställberg-Stenhagen, S., and Stenhagen, E., *J. Biol. Chem.*, **148**, 685 (1943).
7. Unterzaucher, J., *Ber. chem. Ges.*, **73**, 391 (1940).
8. *Cf.* Adam, N. K., The physics and chemistry of surfaces, Oxford, 2nd edition (1938).
9. Andersson, K. J. I., Ställberg-Stenhagen, S., and Stenhagen, E., Svedberg jubilee volume, Upsala, 11 (1944).
10. Teorell, T., and Stenhagen, E., *Biochem. Z.*, **299**, 416 (1938).
11. Schulman, J. H., and Hughes, A. H., *Proc. Roy. Soc. London, Series A*, **138**, 430 (1932).
12. Bernal, J. D., and Crowfoot, D., *Tr. Faraday Soc.*, **29**, 1032 (1933).
13. Francis, F., and Piper, S. H., *J. Am. Chem. Soc.*, **61**, 577 (1939).
14. Müller, A., *Proc. Roy. Soc. London, Series A*, **138**, 514 (1932).
15. *Cf.* Smith, J. C., *Ann. Rep. Progress Chem.*, **35**, 258 (1938).
16. *Cf.* Malkin, T., *Tr. Faraday Soc.*, **29**, 977 (1933).

PHENYL PANTOTHENONE, AN ANTAGONIST OF PANTOTHENIC ACID

By D. W. WOOLLEY and M. L. COLLYER

(*From the Laboratories of The Rockefeller Institute for Medical Research, New York*)

(Received for publication, April 3, 1945)

It has recently been shown that the ketone analogues of nicotinic acid (1) and of *p*-aminobenzoic acid (2) caused signs of deficiency of these vitamins in animals and bacteria, and that these effects of the ketones were overcome by simultaneous administration of increased quantities of the structurally related vitamins. It was therefore of interest to determine whether the exchange of a ketone group for a carboxyl group was a general method for the conversion of metabolites into inhibitory structural analogues (3).

To this end ketone analogues of pantothenic acid have been synthesized and tested for their ability to cause signs of pantothenic acid deficiency. N-(α,γ-Dihydroxy-β,β-dimethylbutyryl)aminoethyl methyl ketone, the methyl ketone analogue, which may be called methyl pantothenone, was deleterious to the growth of bacteria, but its effects were not erased by pantothenic acid. The corresponding phenyl ketone, however, did produce reversible inhibition of microbial growth. This substance, which was N-(α,γ-dihydroxy-β,β-dimethylbutyryl)aminoethyl phenyl ketone, was called phenyl pantothenone.

The synthesis of phenyl pantothenone presented some difficulties, since aminoethyl phenyl ketone which should condense with the lactone moiety of pantothenic acid to yield the desired product was stable only as the hydrochloride. The free amino ketone readily condensed with itself to yield both quinoline derivatives and chain polymers. Three different schemes were devised to circumvent this obstacle and arrive at the desired ketone. In the first method, aminoethyl phenyl ketone hydrochloride was allowed to react with an excess of α-hydroxy-β,β-dimethylbutyrolactone for a short period in alcoholic sodium hydroxide and the mixture of substances which resulted was separated. Although this procedure gave rather poor yields, it was the most convenient of the three modes of synthesis, since it provided pure phenyl pantothenone without recourse to elaborate purification. The second method of synthesis consisted of conversion of aminoethyl phenyl ketone hydrochloride to its *p*-nitrophenylhydrazone, condensation of the latter with the necessary lactone, and hydrolysis of the phenyl pantothenone *p*-nitrophenylhydrazone to phenyl pantothenone. This procedure was unsuitable because of the

difficulty with which the first hydrazone reacted with the lactone, and more especially because of the ease with which the amide linkage of the phenyl pantothenone was split during hydrolysis of the second hydrazone. The third method of synthesis involved the conversion of pantothenic acid to the corresponding diacetyl acid chloride, the condensation of this compound with benzene in the presence of aluminum chloride, and the removal of the acetyl radicals by differential hydrolysis. The yields by

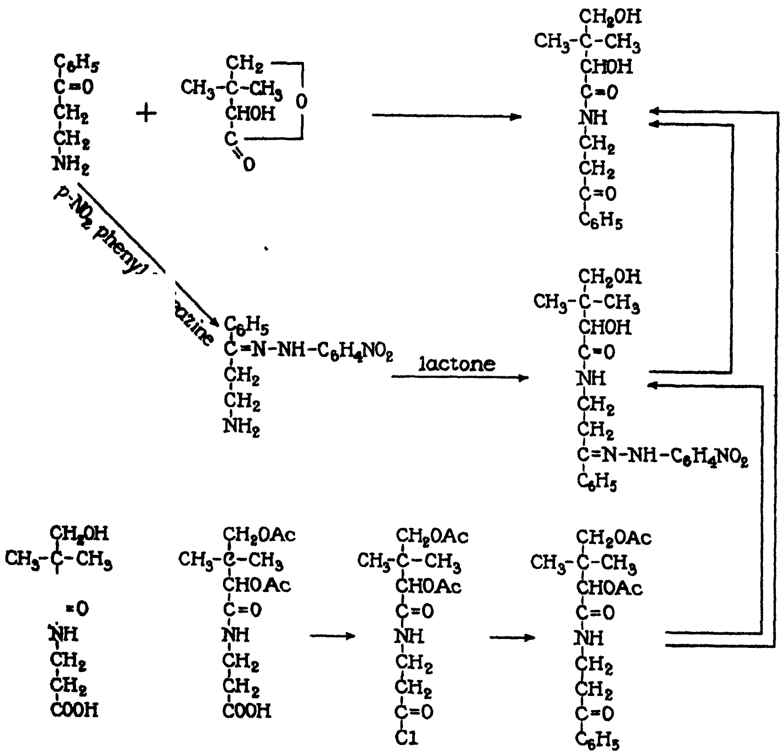


FIG. 1. Routes of synthesis of phenyl pantothenone

this method were good, but the product was contaminated with a non-nitrogenous material high in carbon which was removed with considerable difficulty. The chemical transformations are depicted in Fig. 1.

The structure of phenyl pantothenone obtained by the first and third methods was confirmed by degradation to aminoethyl phenyl ketone hydrochloride and α-hydroxy-β,β-dimethylbutyrolactone.

EXPERIMENTAL

Methyl Pantothenone—Aminoethyl methyl ketone hydrochloride was prepared from β-alanine according to the method of Keil (4). This was

refluxed in methanol for 1 hour with 1 equivalent of *l*-α-hydroxy-β,β-dimethylbutyrolactone[1] and 1 equivalent of N methanolic NaOH. A slight excess of N HCl was added to the cooled solution and the mixture was concentrated to half its volume under reduced pressure, and extracted with butanol. The butanol extracts when dried and concentrated left a liquid ketone. The material was not pure as judged by nitrogen content (found 7.0, calculated 6.5), but further purification was not possible. In view of the type of biological activity of the compound (see below) the synthesis was not investigated further. However, since an analogous reaction with aminoethyl phenyl ketone hydrochloride was shown to yield phenyl pantothenone, it was assumed that methyl pantothenone was contained in the preparation.

Phenyl Pantothenone. From Aminoethyl Phenyl Ketone Hydrochloride—1.85 gm. of aminoethyl phenyl ketone hydrochloride, prepared from β-alanine (5), and 1.50 gm. of *l*-α-hydroxy-β,β-dimethylbutyrolactone were dissolved in 10 cc. of absolute methanol. The solution was heated to boiling and slowly treated with 10 cc. of N NaOH in absolute methanol. The mixture was refluxed for half an hour, cooled, treated with 15 cc. of aqueous N HCl, concentrated under reduced pressure to about 10 cc., and diluted with 15 cc. of water. The resulting mixture was cooled and filtered from the crystals which had separated.[2] The filtrate was extracted four times with ethyl acetate, and the extracts were dried with $MgSO_4$ and freed of solvent under reduced pressure. The residue which remained was taken up in 50 cc. of absolute ether, filtered, and the ether-soluble portion was shaken out with 50 cc. of cold N NaOH to remove unchanged lactone. The either phase was immediately dried with $MgSO_4$, and freed of ether under reduced pressure. 400 mg. of a liquid were obtained.

$C_{15}H_{21}O_4N$. Calculated, C 64.5, H 7.53; found, C 64.8, H 7.54

When a 20 per cent solution in alcohol was stored for several days, white crystals slowly formed. These melted at 126°. The compound was moderately soluble in water and very soluble in alcohol. When it was mixed with 1 equivalent of *p*-nitrophenylhydrazine in alcohol solution, and

[1] We wish to thank Dr. R. T. Major of Merck and Company, Inc., for gifts of this compound.

[2] The white needles which formed at this point melted at 192° and gave analyses which corresponded to the formula $C_{25}H_{27}O_5N$. This compound was quite stable to acid hydrolysis. It was inhibitory to the growth of all bacterial species used in this work but about 1 mg. per cc. was required for half maximal effect. Towards the two yeasts employed it was quite toxic (reduced growth to half the maximum at 3 γ per cc. for *Saccharomyces cerevisiae* and at 2 γ for *Endomyces vernalis*). The inhibition of growth was not relieved by pantothenic acid. The high activity against yeasts was shown to be associated largely with the low pH of the basal medium used for these organisms.

water was added, a hydrazone separated. If the dilution with water was not carried out very slowly, the hydrazone formed as an oil which solidified only with difficulty. The derivative began to soften at 110° and melted at 118°.

$C_{21}H_{26}O_5N_4$. Calculated, N 13.5; found, N 13.2

From Aminoethyl Phenyl Ketone p-Nitrophenylhydrazone—1.85 gm. of aminoethyl phenyl ketone hydrochloride dissolved in 4 cc. of alcohol were added to a solution of 1.53 gm. of *p*-nitrophenylhydrazine in 10 cc. of warm alcohol plus 2 drops of acetic acid. The solution was quickly treated with 1 gm. of anhydrous sodium acetate in 10 cc. of water. The crystals which separated during storage in the cold were collected and recrystallized from alcohol. 1.9 gm.; m.p. 241–243°.

$C_{15}H_{16}O_2N_4$. Calculated, N 19.7; found, N 19.7

1 gm. of the above hydrazone and 2 gm. of *l*-α-hydroxy-β,β-dimethylbutyrolactone were sealed in a tube and heated for 3 hours in an oil bath at 135°. The reaction mixture was then extracted with 50 cc. of ethanol. 430 mg. of unchanged aminoethyl phenyl ketone *p*-nitrophenylhydrazone, m.p. 243°, remained undissolved. The alcohol extract was concentrated under reduced pressure and diluted with an equal volume of water. Here, as in the preparation of phenyl pantothenone *p*-nitrophenylhydrazone described above, it was necessary to add the water slowly if formation of an oily precipitate was to be avoided. The hydrazone was recrystallized and found to melt at 118° after softening at 109°.

No very satisfactory method was encountered for the hydrolysis of the hydrazone to phenyl pantothenone, but small amounts of biologically active material were obtained by refluxing a solution of the hydrazone in acetone plus 1 drop of concentrated HCl for 15 minutes. The *p*-nitrophenylhydrazone of acetone (m.p. 144°) was separated from the phenyl pantothenone by concentration of the solution and extraction with ether. The phenyl pantothenone was obtained in very small yield as a yellow liquid. Attempts to regenerate phenyl pantothenone by treatment of the hydrazone with benzaldehyde and benzoic acid in aqueous suspension were not successful.

From Pantothenic Acid—5 gm. of calcium *d*-pantothenate were dissolved in water and freed of calcium ion exactly with oxalic acid. The solution of pantothenic acid was then concentrated[3] at low temperature to dryness. The residual syrup was dissolved in 50 cc. of acetic anhydride plus 10 cc. of dry pyridine and allowed to stand overnight. The excess reagents were

[3] All evaporations described in this section were carried out under reduced pressure.

removed under reduced pressure, and the acetylation was repeated. The excess reagents were again removed and the last traces were distilled off with anhydrous benzene. The syrupy residue was dissolved in 50 cc. of dry ether and allowed to react at room temperature with 5 gm. of PCl_5. The excess reagent was filtered off and the filtrate was evaporated. The residue was dissolved in dry benzene, concentrated to dryness, and dissolved in 50 cc. of dry benzene. The solution was treated with 5 gm. of $AlCl_3$, and the mixture was refluxed for an hour. The cooled reaction product was shaken with 100 cc. of N HCl, and the organic phase was diluted with ether and extracted with water, then with sodium carbonate solution (to remove unchanged pantothenic and acetyl pantothenic acids), and dried with $MgSO_4$. After the solvent was removed, the residue was dissolved in 50 cc. of N methanolic NaOH, and allowed to stand at room temperature for 2 hours. 60 cc. of N aqueous HCl were added and the methanol was removed under reduced pressure. The aqueous solution was then extracted four times with ethyl acetate, the extracts were dried with $MgSO_4$, and the preparation was freed of solvents. 3.6 gm. of a brownish oil were obtained. Although the per cent of C was too high and the N too low for this material to be pure phenyl pantothenone, the preparation was biologically active against *Lactobacillus casei* and *Endomyces vernalis* in amounts nearly the same as the effective doses of pure phenyl pantothenone. A difficultly crystallizable *p*-nitrophenylhydrazone was obtained from it which softened at 110° and melted at 116°. Finally, acid hydrolysis (see below) led to the isolation of aminoethyl phenyl ketone hydrochloride and α-hydroxy-β,β-dimethylbutyrolactone in good yield.

Hydrolysis of Phenyl Pantothenone—560 mg. of pure phenyl pantothenone obtained by the first method of synthesis were dissolved in 25 cc. of N HCl in 50 per cent alcohol, and the solution was refluxed for 2 hours. The alcohol was removed under reduced pressure, and the aqueous solution was filtered to remove a small amount of oil which had separated. It was then extracted four times with ethyl acetate, and the extract was dried and evaporated. 240 mg. of long needles were obtained which, after recrystallization from ether-petroleum ether, melted at 87°. The melting point was not depressed when the compound was mixed with authentic *l*-α-hydroxy-β,β-dimethylbutyrolactone.

The aqueous residue from the ethyl acetate extraction was concentrated under reduced pressure and the residue was crystallized from alcohol. 320 mg. of white crystals were obtained which melted at 109°, and at the same temperature when mixed with authentic aminoethyl phenyl ketone hydrochloride. The *p*-nitrophenylhydrazone was sparingly soluble in alcohol and melted at 238°.

Similar operations performed on 740 mg. of the crude phenyl pantothenone obtained by the third method of synthesis yielded 172 mg. of the lactone which melted at 89°.

$C_6H_{10}O_3$. Calculated, C 55.4, H 7.7; found, C 55.5, H 7.7

340 mg. of aminoethyl phenyl ketone hydrochloride, m.p. 107°, and *p*-nitrophenylhydrazone, m.p. 243°, were also obtained.

Method of Microbiological Tests—All of the bacterial species were cultivated in the highly purified pantothenic acid-free medium of Landy and Dicken (6). For the yeasts, the synthetic basal medium described previously (7) was used, except that thiamine was added and pantothenic acid was omitted. The inoculum in all instances was prepared by washing cells from a vigorously growing culture of the organisms with buffer, and diluting 50-fold. Graded amounts of pantothenic acid and of phenyl pantothenone were added to a series of tubes of basal medium; the tubes were sterilized in an autoclave at 15 pounds pressure for 15 minutes, cooled, and inoculated. The yeasts were grown in 25 cc. Erlenmeyer flasks and the bacteria were cultured in test-tubes. In all cases the final volume of medium in each tube or flask was 5 cc. Incubation was conducted at 37° (30° for *Saccharomyces cerevisiae* and *Lactobacillus arabinosus*) until good growth was obtained (24 to 48 hours, depending on the organism). Growth was then estimated turbidimetrically in the usual way in an Evelyn photoelectric colorimeter. The results were also checked by measurement of the pH of the cultures in the cases of those species which produced acid. Curves were drawn relating growth to amount of phenyl pantothenone added at a given constant level of pantothenic acid, and from these curves the amount of agent necessary to reduce growth to half maximum was determined. Graded amounts of phenyl pantothenone were tested at each of several levels of pantothenic acid, the highest of which was always sufficient to insure that any reversal of the inhibition would be readily apparent. This amount was usually 100 γ per cc.

Inhibition of Microbial Growth with Phenyl Pantothenone—As can be seen from the data in Table I, phenyl pantothenone was active in the prevention of growth of *Lactobacillus casei*. Table II will show that all microbial species tested were inhibited in growth by the compound.

Reversal of Inhibitory Effect with Pantothenic Acid—It can be seen from the data in Table I that the inhibition of growth of *Lactobacillus casei* was relieved by an increase in the amount of pantothenic acid in the medium. This was also found to be true for *Lactobacillus arabinosus* and for hemolytic streptococcus strain 0-90. Both of these species required pantothenic acid as a growth factor in the medium. For the other forms, with the

exception of *Staphylococcus aureus*,[4] which did not need pantothenic acid preformed, the inhibition of growth was not influenced by increases in the

TABLE I

Inhibition of Growth of Lactobacillus casei by Phenyl Pantothenone and Its Reversal with Pantothenic Acid

Pantothenic acid	Phenyl pantothenone	Colorimeter reading
γ per cc.	γ per cc.	
0.04	0	37
0.20	0	36
0.04	110	90
0.04	66	85
0.04	44	40
0.04	22	34
0.08	110	79
0.08	66	58
0.08	44	40
0.20	110	43

TABLE II

Amounts of Phenyl Pantothenone Required to Reduce Growth of Various Microorganisms to Half Maximum in Presence of 0.04 γ of Pantothenic Acid per Cc.

Organism	Phenyl pantothenone	Reversal by pantothenic acid	Pantothenic acid requirement
	γ per cc.		
Lactobacillus casei	54	Yes	Required
" *arabinosus*	180	"	"
Hemolytic streptococcus, strain 0-90 (Group B)	60	"	"
Escherichia coli	2000*	No	Not required
Staphylococcus aureus	140	Yes	" " but slightly stimulatory
Saccharomyces cerevisiae	33	No	Required but replaceable by β-alanine
Endomyces vernalis	39	"	Not required

* Complete inhibition of growth was not obtained with this organism.

pantothenic acid content of the medium. In this connection it is well to note that *Saccharomyces cerevisiae*, which can use β-alanine in place of pantothenic acid, belonged to the second category.

[4] The strain of *Staphylococcus aureus* grew well without pantothenic acid, but was stimulated to grow appreciably more by this vitamin. The effect of phenyl pantothenone was completely reversed by pantothenic acid.

Stimulation of Growth by Phenyl Pantothenone—Subinhibitory amounts of phenyl pantothenone caused slight, but consistent, increases in growth over the controls of several of the organisms examined. This was similar to the stimulatory effects observed with other inhibitory analogues of metabolites (7, 8).

Effects of Aminoethyl Phenyl Ketone—Since the structural alteration involved in passing from pantothenic acid to phenyl pantothenone was in the β-alanine portion of the molecule, it was of interest to determine the growth-inhibitory action of aminoethyl phenyl ketone. This was especially true in the case of *Saccharomyces cerevisiae* for which β-alanine sufficed as a growth factor. Although all of the microbial species tested were inhibited in growth when the ketone was added to the media before sterilization by autoclaving, this effect was shown to be due to the formation of a harmful material by interaction of the constituents of the basal media with the ketone at high temperature. When the ketone was added aseptically after autoclaving, it was not deleterious to growth in concentrations similar to those of phenyl pantothenone. The inhibition of growth observed with the media which had been autoclaved with the aminoethyl phenyl ketone hydrochloride was not reversed by either pantothenic acid or β-alanine.

Growth-Inhibitory and Growth-Stimulatory Action of Crude Methyl Pantothenone—The impure preparation of methyl pantothenone reduced growth of *Lactobacillus casei* and *Saccharomyces cerevisiae* to half maximum at 0.1 to 0.5 mg. per cc. of medium, but this action was not reversed by increased pantothenic acid. On the other hand, when assayed for pantothenic acid activity with either *Lactobacillus casei* or *Saccharomyces cerevisiae* the material was found to be 1 per cent as active as the vitamin.

DISCUSSION

The action of phenyl pantothenone in causing reversible inhibition of microbial growth when viewed in conjunction with the effects observed with 3-acetylpyridine and *p*-aminoacetophenone (1, 2) makes it seem probable that the exchange of a ketone for a carboxyl group is a general method for the conversion of metabolically active compounds into antagonistic agents (3). These agents owe their action at least in part to the production of deficiency of the metabolites to which they bear structural relationship. In the cases of nicotinic acid and *p*-aminobenzoic acid, antagonistic compounds were produced by the substitution of methyl ketone groups for the —COOH. When a similar change was applied to pantothenic acid (to give methyl pantothenone), little success was had. It may be of value to note that in the two former instances the ketone is attached to a negative, aromatic nucleus which may confer certain acidic

properties on the ketone, while in the latter instance the methyl pantothenone has no adjacent negative group. Therefore the introduction of a negative group, as in the case of phenyl pantothenone, seemed desirable. This was the reasoning which led to the trial of this latter compound.

The reversal of the inhibitory action of phenyl pantothenone by pantothenic acid only in those organisms for which the vitamin is a nutritive essential is noteworthy.[5] With several antagonistic analogues, an effect is observed only in the case of organisms which require the related metabolite preformed in the medium. Here, however, is an inhibitory analogue which was harmful to all species tested, but which was antagonized by the related vitamin only with those forms for which the vitamin was a growth factor. A similar situation has been recorded with glucoascorbic acid in animals. This substance caused a disease resembling scurvy in all species examined, but the effects of the agent were counteracted by ascorbic acid only in guinea pigs. This latter species was the only one which required the vitamin in the diet (9, 10).

SUMMARY

Phenyl pantothenone, N-(α,γ-dihydroxy-β,β-dimethylbutyryl)aminoethyl phenyl ketone, the phenyl ketone analogue of pantothenic acid, has been synthesized by three separate procedures, and its structure confirmed by degradation to known compounds. This substance produced inhibition of growth of all microbial species tested. The action was reversed by increased pantothenic acid in the medium, but in general, only in the cases of organisms which required the vitamin preformed in the medium.

BIBLIOGRAPHY

1. Woolley, D. W., *J. Biol. Chem.*, **157**, 455 (1945).
2. Auhagen, E., *Z. physiol. Chem.*, **274**, 48 (1942).
3. Woolley, D. W., *Science*, **100**, 579 (1944).
4. Keil, W., *Z. physiol. Chem.*, **207**, 248 (1932).
5. Gabriel, S., *Ber. chem. Ges.*, **41**, 242 (1908).
6. Landy, M., and Dicken, D. M., *J. Lab. and Clin. Med.*, **52**, 106 (1943).
7. Woolley, D. W., and White, A. G. C., *J. Exp. Med.*, **78**, 489 (1943).
8. Woolley, D. W., *J. Biol. Chem.*, **152**, 225 (1944).
9. Woolley, D. W., and Krampitz, L. O., *J. Exp. Med.*, **78**, 339 (1943).
10. Woolley, D. W., *Federation Proc.*, **3**, 97 (1944).

[5] As noted earlier (foot-note 4), *Staphylococcus aureus* was an exception to this generalization.

THE MICROBIOLOGICAL DETERMINATION OF AMINO ACIDS*

II. ASSAY AND UTILIZATION OF GLUTAMIC ACID AND GLUTAMINE BY LACTOBACILLUS ARABINOSUS

By LUCILE R. HAC, ESMOND E. SNELL, AND ROGER J. WILLIAMS

(From the Amino Products Division of the International Minerals and Chemical Corporation, Toledo, Ohio, and The University of Texas, Biochemical Institute, and the Clayton Foundation for Research, Austin)

(Received for publication, February 27, 1945)

In a discussion of the analytical data available on proteins, Vickery (2) in 1941 stated that few values for glutamic and aspartic acids were worthy of serious consideration. Since that time, however, several promising methods for the chemical analysis of glutamic acid have appeared. Chibnall and coworkers (3, 4), by improved methods of isolation, have reported values considered to be accurate within 2 per cent. Determinations have also been made by chromatographic adsorption (5, 6), by isotope dilution (7), by enzymatic methods (8, 9), by calculation from electrometric titration and gasometric ninhydrin data (10), and by conversion to pyrrolidonecarboxylic acid (11). Although these methods are applicable to the study of pure proteins, they were too tedious and time-consuming for many routine applications. Microbiological methods of assay (1,12–14) which seemed to offer more assurance of usefulness in such studies were, therefore, undertaken.

Since initiation of this work, Dunn *et al.* (14) and Lyman *et al.* (15) have described microbiological methods for the determination of glutamic acid with a basal medium composed of amino acids, vitamins, purine bases, and salts. Lewis and Olcott (16) used a similar technique except that a supplemented "glutamic acid-free casein hydrolysate" was substituted for the amino acid mixture. In each instance the assay organism was *Lactobacillus arabinosus*. All of these workers found the utilization of glutamic acid by this microorganism to be somewhat unusual and its assay slightly more difficult than that of other acids previously reported. It therefore seemed worth while to present additional data pertinent to this method.

Materials and Methods

Microorganism—*Lactobacillus arabinosus* 17-5 was used for all assays. Other microorganisms studied were *L. pentosus* 124-2, *L. casei*, and *Leuconostoc mesenteroides* P-60.

* For Paper I of this series, see McMahan and Snell (1).

Inoculum—The stock stab cultures were maintained on yeast extract-glucose agar (1 per cent glucose, 1 per cent yeast extract, and 1.5 per cent agar) as previously described (1). The inoculum for assay was grown in a medium which contained per liter 5 gm. of Bacto-peptone, 1 gm. of Bacto-yeast extract, 10 gm. of sodium acetate, 10 gm. of glucose, and 5 ml. each of inorganic Salt Solutions A and B. Daily transfers were made serially from the broth with but weekly reference to the stock culture. An 18 to 24 hour culture was centrifuged, washed once with physiological saline, and resuspended in 10 ml. of saline. 1 drop of this suspension per 2.5 ml. of assay medium was considered a "heavy" inoculum. When reference is made to a "light" inoculum, 3 to 7 drops of the above "heavy" suspension were added to 10 ml. of saline. 1 drop of this material, which was just faintly turbid, was used per 2.5 ml. of assay medium.

Basal Medium—The composition of the basal assay medium and the method of preparation of the stock solutions are given in Table I. This medium served as a basic one for study of the assay of several amino acids and differs from that of McMahan and Snell (1) primarily in the increased concentration of vitamins. Certain modifications in amino acid content which have been used in the assay of glutamic acid are also indicated.

Standard Solution—Pure $l(+)$-glutamic acid was used for all the standard curves.

Preparation of Samples—Thick walled Pyrex test-tubes which contained 100 mg. of sample and 2 ml. of 10 per cent hydrochloric acid were sealed in an oxygen flame and autoclaved at 15 pounds pressure for 4 to 36 hours. The tubes were cooled, opened, and the hydrolysates washed out with water. The solution was adjusted with sodium hydroxide to pH 6.1 to 6.3 and diluted to the desired volume, usually 50 or 100 ml.

Procedure—For assay, 125 ml. of the amino acid solution, 1 ml. of the vitamin supplement, 2.5 ml. of Salts A, and 2.5 gm. of glucose constituted a double strength medium. This amount was sufficient for 100 assay tubes if determinations were made turbidimetrically (final volume 2.5 ml.) or 50 tubes if analyses were made titrimetrically (final volume 5 ml.). Ordinarily the amount of sample to be analyzed or the standard solution used was small enough so that its volume (less than 0.2 ml. per 2.5 ml. of medium) could be disregarded. The sample was pipetted into the bottom of the tube[1] and 2.5 ml. of basal medium were added. If it was necessary to use samples larger than 0.2 ml., the volume in each tube was adjusted with water to 1.2 ml. and 1.2 ml.[2] of the double strength basal medium

[1] Serological Kahn pipettes graduated in 0.001 ml. were used for this purpose. The end of the pipette was drawn out to a fine capillary. Such pipettes could be operated easily and accurately with the finger.

[2] Kolmer pipettes designed for use in the complement fixation test and graduated in 1.2 ml. were used.

TABLE I

Complete Basal Medium; Concentration per Ml. of Final Medium

Amino acids		Purines and pyrimidine	
	mg.		*mg.*
l(+)-Arginine monohydrochloride	0.1	Adenine sulfate	0.01
l(−)-Cystine	0.1	Guanine hydrochloride	0.01
Glycine	0.1	Uracil	0.01
l(−)-Histidine monohydrochloride	0.1	Xanthine	0.01
l(−)-Hydroxyproline	0.1		
l(−)-Leucine	0.1	Salts A	
l(+)-Lysine monohydrochloride	0.1	KH_2PO_4	1.0
l(−)-Proline	0.1	K_2HPO_4	1 0
l(−)-Tryptophane*	0.1		
l(−)-Tyrosine	0.1		
l(+)-Aspartic acid	0.4		
l(+)-Glutamic acid†	1 0	Salts B	
dl-Alanine	0 2	$MgSO_4\cdot 7H_2O$	0.2
dl-Isoleucine	0.2		
dl-Methionine*	0.2	$FeSO\cdot 7H_2O$	0.01
dl-Norleucine*	0.2	$MnSO_4\cdot 4H_2O$	0.01
dl-Norvaline*	0 2		
dl-Phenylalanine*	0 2	NaCl	0 01
dl-Serine*	0.2		
dl-Threonine	0.2		
dl-Valine	0 2	$(NH_4)_2SO_4$	3.0
Vitamins			
	γ		
Biotin	0.001	Sodium acetate (anhydrous)	6.0
Folic acid	0.01	Glucose	10.0
Calcium pantothenate	0.2		
Nicotinic acid	0.2		
Riboflavin	0.2		
p-Aminobenzoic acid	0.3		
Thiamine hydrochloride	0.5		
Choline chloride	2.5		
Inositol	2.5		
Pyridoxine hydrochloride‡	10.0		

Stock solutions of the constituents were prepared as follows: Salts A contained 25 gm. each of KH_2PO_4 and K_2HPO_4 per 250 ml. of solution; Salts B contained 10 gm. of $MgSO_4\cdot 7H_2O$, and 0.5 gm. each of $FeSO_4\cdot 7H_2O$, $MnSO_4\cdot 4H_2O$, and NaCl per 250 ml. of solution. The vitamin supplement contained 12.5 γ of biotin, 125 γ of folic acid, 2.5 mg. each of calcium pantothenate, nicotinic acid, and riboflavin, 3.8 mg. of *p*-aminobenzoic acid, 6.3 mg. of thiamine hydrochloride, 31.4 mg. each of choline chloride and inositol, and 125 mg. of pyridoxine hydrochloride dissolved by warming gently in 50 ml. of distilled water. Amino acid mixture, 1 liter of amino acid solution contained 400 mg. each of the *dl*-amino acids, 800 mg. of *l*(+)-aspartic acid, and 200 mg. each of the other *l*-amino acids; 20 mg. each of adenine sulfate,

TABLE I—*Concluded*

guanine hydrochloride, xanthine, and uracil; 6 gm. of ammonium sulfate; and 12 gm. of anhydrous sodium acetate. The materials were dissolved by boiling in about 900 ml. of water. After the solution had cooled, 10 ml. of Salts B were added, the pH of the solution was adjusted to 6.3 with sodium hydroxide, and the final volume adjusted to 1 liter. Such solutions were stored in the refrigerator, usually without preservative, but a thin layer of toluene may be used if necessary.

* Half the indicated amounts have been found satisfactory in assay of glutamic acid.

† Omitted in medium for assay of glutamic acid.

‡ One-tenth the amount indicated is sufficient for assay with *Lactobacillus arabinosus* 17-5.

were added. For titration, double amounts of sample and medium were used. The tubes were capped with metal caps or well fitted glass vials (3 cm. long) and autoclaved at 15 pounds pressure for 15 minutes. After inoculation, the tubes were incubated at 34° for 72 hours.

For turbidity readings the contents of the tubes were diluted with 5 ml. of water delivered from an automatic pipette. A thermocouple turbidimeter (17) was used almost exclusively in this study, but the Klett-Summerson colorimeter also gave satisfactory results.

Titration of the acid produced in the 5 ml. assay quantities was carried out with 0.05 N sodium hydroxide with a glass electrode or brom-thymol blue as an indicator.

The assay range used was 0.01 to 0.2 mg. of $l(+)$-glutamic acid per 2.5 ml. of medium. Final analytical values are the average of duplicate analyses at three or four levels of concentration.

Results

Standard Curve—Typical standard assay curves for glutamic acid are plotted in Fig. 1 with four test organisms, *Lactobacillus arabinosus*, *L. pentosus*, *L. casei*, and *Leuconostoc mesenteroides*. As others have noted with *L. arabinosus* (15, 16), the shape of these curves differs from that reported for other amino acids. There is an initial plateau at which no growth above that of the blank occurs. Once growth is initiated, however, the curves break sharply and rise very rapidly, followed by a more gradual rise proportional to the amount of amino acid present. This latter portion of the curve was used for assay since it gave the most regular and reliable results. A fundamental similarity in assimilation of this amino acid by the four microorganisms is indicated by their growth curves which differ primarily in the concentration of glutamic acid at which growth is initiated.

This inability to utilize glutamic acid at low levels of concentration suggested that it was not itself assimilated by these organisms, but was slowly converted by them into a substance which was assimilable. Early

in this investigation glutamine and glutamic acid were found to have equal activity for *Lactobacillus arabinosus* at relatively high concentrations, but at low levels at which no growth was observed with glutamic acid, glutamine was active. This observation suggested that glutamine rather

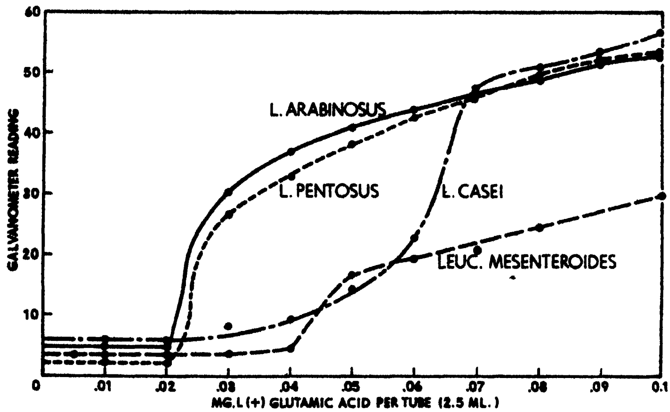


FIG. 1. Comparative response of *Lactobacillus arabinosus*, *L. pentosus*, *L. casei*, and *Leuconostoc mesenteroides* to $l(+)$-glutamic acid. Turbidimetric measurements.

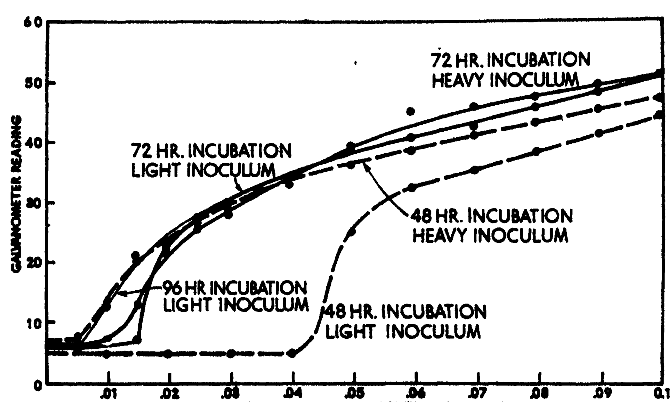


FIG. 2. The effect of the size of inoculum and the period of incubation on the response of *Lactobacillus arabinosus* to $l(+)$-glutamic acid. Turbidimetric measurements.

than glutamic acid might be the substance required for the growth of these microorganisms and that the initial plateau observed in the glutamic acid curve represented a region in which concentration of glutamic acid was too low to permit significant conversion to glutamine by the test organism. Recently Lyman *et al.* (15) have advanced a similar view and have suggested

that small amounts of glutamine be added to the basal medium used for assay of glutamic acid.

If conversion to glutamine or some other more readily assimilable substance is necessary before rapid growth can take place, such conversion

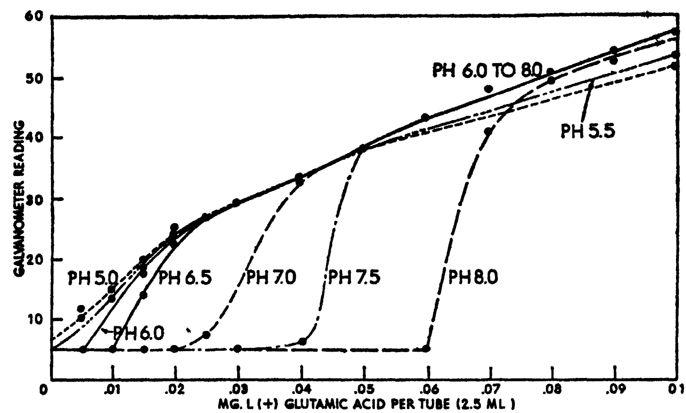


FIG. 3. The effect of initial pH of the basal medium on the response of *Lactobacillus arabinosus* to $l(+)$-glutamic acid. Turbidimetric measurements.

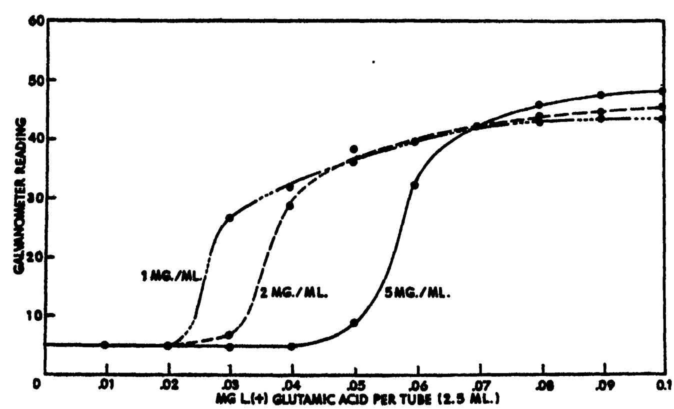


FIG. 4. Effect of varying the concentration of phosphates in the basal medium on the response of *Lactobacillus arabinosus* to $l(+)$-glutamic acid. Turbidimetric measurements.

should be favored by increasing the size of the inoculum or the period of incubation. From Fig. 2 it is evident that growth of *Lactobacillus arabinosus* occurred at lower concentrations of glutamic acid when a large inoculum or a relatively long period of incubation was used.

Since growth of these organisms is accompanied by acid production, the effect of variation in the pH of the basal medium upon growth response

to glutamic acid was determined. As the initial pH of the medium was decreased, growth was initiated at lower and lower concentrations of glutamic acid until at pH 5.0 the initial plateau of the growth curve was almost completely eliminated (Fig. 3). In such a medium at high con-

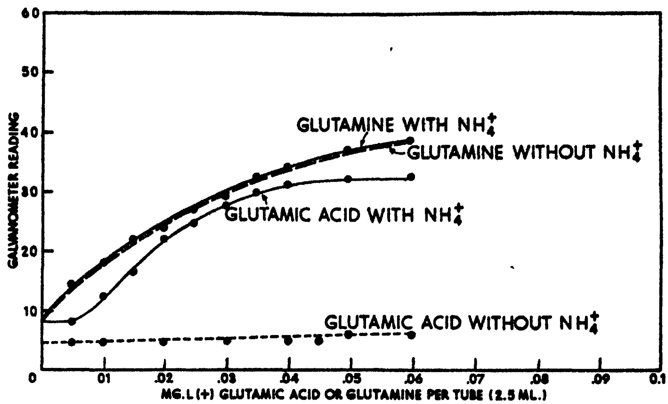


FIG. 5. Comparative response of *Lactobacillus arabinosus* to glutamine and $l(+)$-glutamic acid in the presence and absence of ammonium sulfate. A "heavy" inoculum was used and the period of incubation was 24 hours.

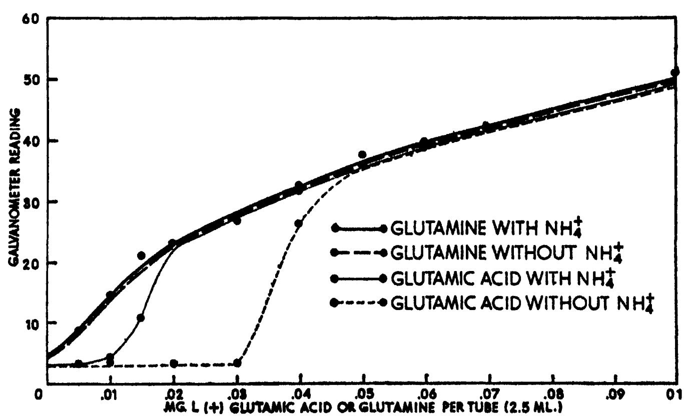


FIG. 6. Comparative response of *Lactobacillus arabinosus* to glutamine and $l(+)$-glutamic acid in the presence and absence of ammonium sulfate. A "light" inoculum was used and the period of incubation was 72 hours.

centrations of glutamic acid, however, maximum growth was not as great as that obtained in mediums of pH 6.0 to 8.0. It might be expected that maximum levels of growth would be lower in a medium of relatively high initial acidity, since growth of these organisms is inhibited by relatively high concentrations of acid. Acid produced as a metabolic product of

growth would reach toxic levels earlier in a medium more acid initially. The pH selected for use in assay, therefore, was 6.8.

It was possible to initiate growth at lower concentrations of glutamic acid in a medium of pH 6.8 if the amount of phosphate in the basal medium was decreased (Fig. 4). This effect was probably due to the fact that smaller amounts of acid were required to shift the pH of the less highly buffered medium to levels permitting more ready conversion of glutamic acid to the assimilated product.

These experiments all tend to indicate that the mechanism for the conversion of glutamic acid to glutamine operates optimally in acidic solutions. Some type of ammonia transfer must also be involved. In order to determine whether the ammonium ion served this function, growth curves

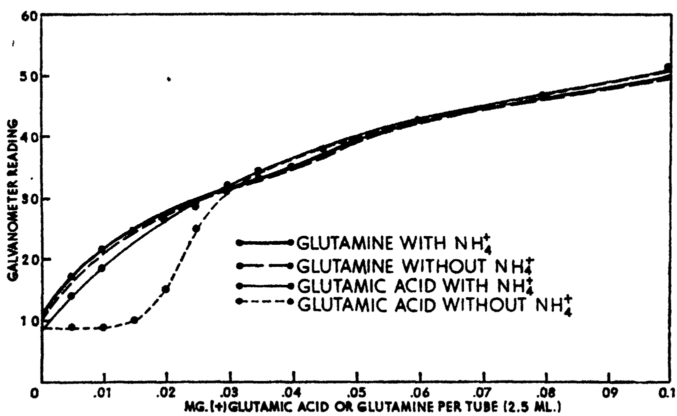


FIG. 7. Comparative response of *Lactobacillus arabinosus* to glutamine and $l(+)$-glutamic acid in the presence and absence of ammonium sulfate. A "heavy" inoculum was used and the period of incubation was 72 hours.

produced by glutamic acid and glutamine were compared with and without ammonium salts added to the basal medium. Both light and heavy inocula were used and readings were made at 24, 48, and 72 hour intervals. The omission of ammonium salts from the basal assay medium had no effect upon the utilization of glutamine, but their absence delayed growth and prevented utilization of glutamic acid at low concentrations. This was particularly noticeable when the period of incubation was short (Fig. 5), or when the inoculum was small (Fig. 6). Even with a heavy inoculum, no growth was evident at 24 hours (Fig. 5). Growth occurred at high concentrations of glutamic acid at 48 hours and at lower concentrations at 72 hours, but the initial plateau at which no growth occurred was more pronounced than when ammonia was present (Fig. 7). The addition of ammonium sulfate to the glutamic acid medium resulted in growth curves

at 24 hours similar to those for glutamine except that the initial plateau was evident and growth was slower throughout the entire curve. At 72 hours, the glutamine and glutamic acid curves coincided except at the very lowest concentration of glutamic acid. These results indicate that

TABLE II

Comparative Growth-Promoting Activities of Glutamic Acid and Related Substances

Test organism, *Lactobacillus arabinosus;* galvanometer readings.

Organism per tube	Glutamine (filtered)	Glutamic acid			α-Ketoglutaric acid	Glutathione	Pyrrolidonecarboxylic acid	α-Hydroxyglutaric acid
		l (+)-	*dl*-	*d*(−)-				
mg.								
0.0	5.0	3.0	4.0	4.0	7.0	9.0	4.0	4.0
0.01	12.0	4.0	4.0	4.0				
0.02	20.0	18.5	4.0	4.0		9.0		
0.04	31.5	30.3	21.5	4.0		24.5		
0.07	38.8	38.4	35.0	4.0		34.2		
0.10	41.5	41.5	43.0	4.0	7.0	43.2	4.0	4.0
0.13	52.0	52.5			12.0			
0.16	57.0	57.0			35.5			
0.20	64.0	63.5		4.0	58.0	59.0	4.0	4.0
0.30	72.2	73.0		9.0	72.0	66.5		
0.40	74.0	76.5		23.5	73.3			
0.50		76.5	75.5	40.5			4.0	4.0
0.60	76.0	76.5		47.0	77.0	76.5		
0.70		77.0		54.8				
0.80	76.0	77.0		60.2				
0.90		77.0		64.5				
1.00	77.0	77.0	77.0	67.0	77.0	77.0	4.0	4.0
2.00				77.0				

0.05 mg. *l*(+)-glutamic acid		35.4
0.05	+ 0.02 mg. α-ketoglutaric acid	38.2
0.05	+ 0.04 " " "	41.0
0.05	+ 0.12 " " "	54.5
0.05	+ 0.02 " pyrrolidonecarboxylic acid	35.0
0.05	+ 0.12 " " "	35.8
0.05	+ 0.02 " α-hydroxyglutaric acid	35.7
0.05	+ 0.12 " " "	34.8

conversion of glutamic acid to glutamine does occur and that ammonium salts are utilized in the conversion.

Identical values were obtained with four different preparations of glutamine which were sterilized by filtration through a Berkefeld filter and added aseptically to the sterile basal medium just before inoculation. Glutamine is rapidly decomposed, primarily to ammonium pyrrolidone-α-carboxylate or pyrrolidonecarboxylic acid (18), by heating it in an aqueous

solution. A similar conversion of glutamic acid takes place in slightly acid solutions, but the reaction is slower (19). Curves obtained with glutamic acid sterilized in the autoclave or by filtration were identical.

Use has been made of these facts in the estimation of the glutamine content of various naturally occurring substances. Samples to be tested were analyzed directly and after hydrolysis. For direct analysis, aliquots of the samples to be tested were sterilized by filtration through a Berkefeld filter, and by autoclaving. The values so obtained compared with those determined after hydrolysis gave at least a rough indication of the proportions of the materials present as glutamine, glutamic acid, and pyrrolidonecarboxylic acid. Refinement of these techniques should result in a

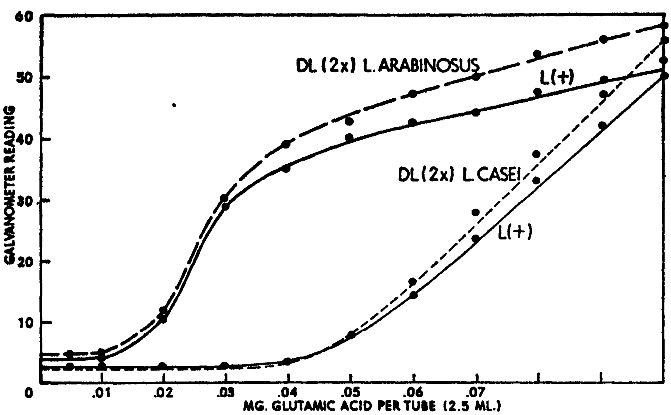


FIG. 8. Comparative response of *Lactobacillus arabinosus* and *Lactobacillus casei* to $l(+)$- and dl-glutamic acids. The concentration of the dl isomer is twice that of the naturally occurring antipode.

fairly accurate method for determining the proportions of these substances present in natural products.

Hydrolyzed samples of glutathione gave theoretical values for glutamic acid. Unhydrolyzed samples of this peptide showed some activity which increased with increasing concentration, but was less than that to be expected from its glutamic acid content.

Several compounds which have a chemical structure similar to glutamine and glutamic acid were tested for their ability to promote growth. In Table II are listed the turbidimetric values for these substances compared with those obtained with glutamine and $l(+)$-glutamic acid.

$d(-)$-Glutamic acid[3] initiated growth at much higher concentrations of glutamic acid than $l(+)$-glutamic acid. The activity of the unnatural

[3] Furnished by Dr. A. C. Kibrick.

TABLE III
*Glutamic Acid Content (in Per Cent) of Various Pure Proteins**

		Hydrolysis time								Values reported in literature	
		0 hr.	4 hrs.	6 hrs.	10 hrs.	16 hrs.	20 hrs.	24 hrs.	36 hrs.	Microbiological	Chemical
Glutamic acid recovery		100	100	100	100	100	100	98	97		
Casein (Labco)	No. of analyses		1	1	10	5	5	8		22.5 (14)	
	Glutamic acid		21.8	22.2	22.7	21.5	21.8	21.7		19.7 (16)	22.0 (2)
										21.5 (15)	
Lactoglobulin†	No. of analyses				4	6	2	3	2		21.5 (3)
	Glutamic acid				17.7	17.9	18.9	18.8	19.6	18.7 (16)	19.0‡
Lactoglobulin§	No. of analyses				3		1	2	2		
	Glutamic acid				18.8		18.9	19.0	18.6		
Egg albumin†	No. of analyses				5		1		2	*	
	Glutamic acid				14.9		15.1		14.1		
Egg albumin‖	No. of analyses				1	1		2	3	13.7 (16)	16.1 (3)
	Glutamic acid				15.0	15.2		15.0	14.8	14.3 (15)	17.0 (10)
Gliadin‖	No. of analyses				1	3	2	2	1		46.9 (2)
	Glutamic acid				45.3	46.3	47.3	45.5	47.3	44.2 (16)	45.7 (10)
Gelatin (Knox)	No. of analyses				2	1	1	1			11.7 (10)
	Glutamic acid				10.7	11.3	11.9	11.2		10.2 (16)	5.8 (20)
Silk fibroin†	No. of analyses							2	2	2.2 (14)	
	Glutamic acid							2.2	2.4	2.1 (16)	3.5 (10)
Edestin (Difco standardized)	No. of analyses							2			18.3 (10)
	Glutamic acid							21.3		19.1 (16)	20.7 (3)
Horse hemoglobin† (recrystallized 3 times)	No. of analyses				1	1	1	3	1		
	Glutamic acid				9.7	8.5	8.8	10.8	12.4		6.3 (21)
Horse carboxyhemoglobin¶ (recrystallized 2 times)	No. of analyses							2	2		6.75 (2)
	Glutamic acid							8.9	9.0		8.5¶

Each analysis is the average of three to six duplicate determinations made at different levels of concentration.

* Values reported in this paper are corrected for moisture content of the protein. When possible, comparative values from the literature are also given on the moisture-free basis; in some cases, however, authors have not been explicit on this point. We are indebted to Beverly M. Guirard for the moisture determinations. † Furnished by the late Dr. Max Bergmann. ‡ Personal communication, Dr. G. L. Foster; analysis by isotope dilution. § Furnished by Dr. Lila Miller. ‖ Furnished by Dr. H. S. Olcott. ¶ Furnished by Dr. G. L. Foster; analysis by isotope dilution, 8.5 per cent glutamic acid.

antipode was evidenced somewhat earlier, however, if a *dl* mixture was used. Several samples of *dl*-glutamic acid were tested and repeated assays were made. All of the samples showed approximately 50 per cent activity at low concentrations, but greater activity at higher concentrations. This

TABLE IV

*Comparison of Values Obtained with Light and Heavy Inoculum**

Hydrolysate	Per cent glutamic acid							
	Casein		Lactoglobulin		Egg albumin		Gliadin	
	Light inoculum	Heavy inoculum	Light inoculum	Heavy inoculum	Light inoculum	Heavy inoculum	Light inoculum	Heavy inoculum
mg.								
0.015	21.00	21.33	18.12	18.75	14.16	15.00	45.00	44.60
0.020	21.25	22.11	17.50	19.62	13.96	14.40	46.25	41.66
0.026	20.19	21.75	17.00	18.12	13.55	13.60	46.50	43.75
Average .	20.8	21.8	17.5	18.8	13.9	14.3	45.9	43.3

* Incubation period 72 hours. Each value is an average of duplicate determinations.

TABLE V

Recovery of Glutamic Acid Added to Protein Hydrolysates

Protein	$l(+)$-Glutamic acid			Per cent recovery
	Found per aliquot	Added	Total	
	mg.	*mg.*	*mg.*	
Casein.....	0.0206	0.02	0.0400	98.6
	0.0206	0.03	0.0510	100.7
	0.0325	0.02	0.0520	99.0
	0.0325	0.04	0.0725	100.0
	0.0440	0.03	0.0740	100.0
Lactoglobulin	0.0272	0.02	0.0470	99.5
	0.0368	0.03	0.0671	100.3
Egg albumin . ..	0.0288	0.03	0.0580	98.6
	0.0429	0.02	0.0622	98.8

increasing activity with increasing concentrations of *dl*-glutamic acid for *Lactobacillus arabinosus* and *Lactobacillus casei* is shown in Fig. 8.

α-Ketoglutaric acid was utilized at lower concentrations than $d(-)$-glutamic acid, but at higher concentrations than $l(+)$-glutamic acid. Here, as in the case of the unnatural antipode, the addition of even small amounts of α-ketoglutaric acid (0.02 mg.) to limiting quantities of $l(+)$-glutamic acid resulted in increased growth (Table II).

Pyrrolidonecarboxylic acid and α-hydroxyglutaric acid were completely inactive.

Reliability of Assay. Agreement with Other Methods of Analysis—The assay results obtained with several pure proteins are listed in Table III. Consistent values were obtained on repeated assay. Average values obtained after 4, 6, 10, 16, 20, 24, and 36 hours hydrolysis are compared with

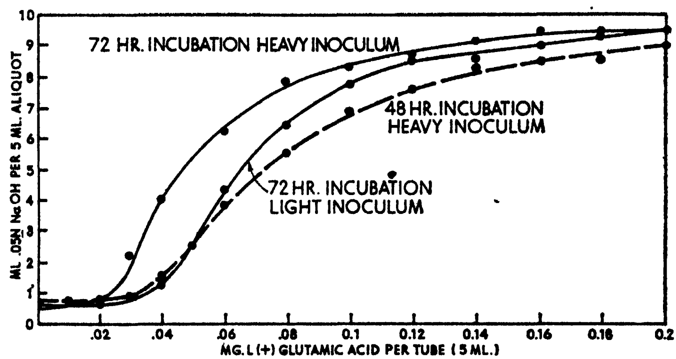


FIG. 9. The effect of size of inoculum and the period of incubation on the response of *Lactobacillus arabinosus* to $l(+)$-glutamic acid. Titrimetric measurements.

TABLE VI

*Comparison of Turbidimetric and Titrimetric Values**

Protein	Per cent glutamic acid	
	Turbidity	Titration
Casein	22.6	20.8
	21.1	21.4
	20.7	20.6
Lactoglobulin	18.7	18.1
Egg albumin	14.4	14.0
Gliadin	44.9	44.0

* Incubation period, 72 hours. Each figure is the average of duplicate determinations made at three or four levels of concentration.

those obtained with pure $l(+)$-glutamic acid similarly treated. The highest values for lactoglobulin, gliadin, and horse hemoglobin were obtained after 36 hours hydrolysis, even though pure $l(+)$-glutamic acid had undergone slight racemization at a similar period. The values obtained are in good agreement with those obtained by similar microbiological techniques, and with the most reliable chemical analyses.

Agreement of Values Calculated at Various Assay Levels—In Table IV

the values obtained at various assay levels are compared when light and heavy inocula were used. There is good agreement at different levels of concentration. Since a fairly heavy inoculum after 72 hours incubation gives slightly higher assay values as well as an increased range for assay, it is preferable to a very light inoculum.

Recovery Experiments—Recovery values for $l(+)$-glutamic acid added to hydrolysates of casein, lactoglobulin, and egg albumin are given in Table V. Although the recovery values obtained with these pure proteins were excellent, certain difficulties were encountered with some industrial materials in which both stimulatory and inhibitory substances were noted.

Turbidity Versus Titration Values—In Fig. 9 are plotted the titrimetric curves which correspond to the turbidimetric curves given in Fig. 2. Values obtained from some of the proteins tested by the two methods are compared in Table VI. The values obtained by turbidimetric measurements are usually slightly higher than those obtained by titration. Since the turbidimetric method was easier and less time-consuming than titration, it was preferred provided the samples were not too highly colored and did not develop turbidity during incubation.

DISCUSSION

All of the data concerned with utilization of glutamic acid are readily explained by the assumption that glutamine, rather than glutamic acid, is the substance actually utilized by the test organism. Glutamine was the most active substance tested, and growth was proportional to the concentration. Conditions which increased the availability of glutamic acid caused the dose-response curve to become more similar to that obtained with glutamine. Such conditions included the use of heavy inocula, extension of the period of incubation, and a decrease in the pH of the medium. Especially suggestive was the fact that omission of ammonium salts greatly reduced the availability of glutamic acid, but failed to affect response to glutamine in any manner.

According to Pollack and Lindner (22), glutamine and glutamic acid had equal growth-promoting activity for *Lactobacillus arabinosus*, *L. pentosus*, *L. casei*, and *Streptococcus lactis*. Results presented above and those of Lyman (15), Lewis and Olcott (16), and Niven (23) fail to confirm these data at low concentrations of glutamic acid. Pollack and Lindner did not consider it likely that glutamic acid was converted to glutamine because the original medium contained no source of ammonia other than amino acids and organic nitrogen compounds. This view is hardly consistent with the known versatility of microorganisms. Although ammonium chloride added to their medium did increase growth obtained with glutamic

acid, they considered the effect to be too small to be accounted for by conversion of glutamic acid to glutamine.

McIlwain *et al.* (24) reported that glutamine could be replaced by high concentrations of *dl*-glutamic acid for the growth of *Streptococcus hemolyticus* and considered that the organism might be able to synthesize glutamine when sufficiently high concentrations of glutamic acid were present. It was suggested that the essential growth activity dependent upon glutamine was ammonia transfer. Later, however, in studies demonstrating the extreme specificity of glutamine for certain microorganisms, McIlwain retracted these suggestions (25).

Lyman *et al.* (15) considered it advisable to eliminate the initial plateau obtained with glutamic acid by the addition to the basal medium of suboptimum quantities of glutamine. In the course of this investigation it was found that small amounts of a tryptic digest of casein also eliminated this initial plateau. Such digests are superior to glutamine for this purpose because they are stable to heat and can be autoclaved with the medium without loss in activity. They have been previously reported (26, 27) to contain substances, probably peptides, which replace glutamine and asparagine for lactic acid bacteria. Addition of such substances to the assay medium produced little or no difference in assay values, and they were therefore not used routinely.

When used alone in high enough concentration, $d(-)$-glutamic acid, α-ketoglutaric acid, and glutathione permitted attainment of the same maximum growth level achieved with glutamine or $l(+)$-glutamic acid, although they were less active than either of these compounds at low levels of concentration. When tested in the presence of suboptimum quantities of $l(+)$-glutamic acid, $d(-)$-glutamic acid and α-ketoglutaric acid showed activity at lower concentrations than when tested alone. This indicates that conversion to glutamine is also necessary for utilization of these compounds, and such conversion occurs at lower concentrations when the test organism is actively growing. This view is at variance with that expressed by Dunn *et al.* (14), who postulated some essential rôle for $d(-)$-glutamic acid on the basis of similar data for *dl*-glutamic acid.

SUMMARY

A turbidimetric or titrimetric method for the quantitative determination of $l(+)$-glutamic acid in protein hydrolysates is described. *Lactobacillus arabinosus* is the test organism. The following values, corrected for moisture, were obtained with the proteins studied after 24 hours hydrolysis: Labco casein 21.7, lactoglobulin 18.8 and 19.0 (different preparations), egg albumin 15.0, gliadin 45.5, gelatin (Knox) 11.2, silk fibroin 2.2, edestin

21.3, horse hemoglobin 10.8, and horse carboxyhemoglobin 8.9 per cent. Recovery experiments, agreement of values calculated at various assay levels, and upon repeated assay, specificity studies, and agreement with other methods of analysis all indicate reliability of the proposed method. Occasional low values as compared with chemical analyses require further study for explanation. In some instances, both stimulatory and inhibitory substances have been encountered in crude natural materials, and further study of their assay is indicated.

Glutamine is more active than $l(+)$-glutamic acid; activity of the latter is increased toward that of glutamine as a limit by increasing the size of the inoculum, lengthening the incubation period, lowering the initial pH of the medium, and adding ammonium salts to the medium. These data indicate that glutamic acid is converted to glutamine before utilization. This is probably also true for $d(-)$-glutamic acid, α-ketoglutaric acid, and glutathione, which are less active than $l(+)$-glutamic acid, but which permit maximum growth at high concentrations. Pyrrolidonecarboxylic acid and hydroxyglutaric acid are inactive.

For some samples, the method appears adaptable to the determination of glutamine as well as glutamic acid.

BIBLIOGRAPHY

1. McMahan, J. R., and Snell, E. E., *J. Biol. Chem.*, **152,** 83 (1944).
2. Vickery, H. B., *Ann. New York Acad. Sc.*, **41,** 87 (1941).
3. Bailey, K., Chibnall, A. C., Rees, M. W., and Williams, E. F., *Biochem. J.*, **37,** 360 (1943).
4. Chibnall, A. C., Rees, M. W., and Williams, E. F., *Biochem. J.*, **37,** 372 (1943).
5. Wieland, T., *Ber. chem. Ges.*, **75,** 1001 (1942).
6. Cannan, R. K., *J. Biol. Chem.*, **152,** 401 (1944).
7. Rittenberg, D., and Foster, G. L., *J. Biol. Chem.*, **133,** 737 (1940).
8. Cohen, P. P., *Biochem. J.*, **33,** 551 (1939).
9. Woodward, G. E., Reinhart, F. E., and Dohan, J. S., *J. Biol Chem.*, **138,** 677 (1941).
10. Kibrick, A. C., *J. Biol. Chem.*, **152,** 411 (1944).
11. Olcott, H. S., *J. Biol. Chem.*, **153,** 71 (1944).
12. Shankman, S., Dunn, M. S., and Rubin, L. B., *J. Biol. Chem.*, **151,** 511 (1943).
13. Kuiken, K. A., Norman, W. H., Lyman, C. M., Hale, F., and Blotter, L., *J. Biol. Chem.*, **151,** 615 (1943).
14. Dunn, M. S., Camien, M. N., Rockland, L. B., Shankman, S., and Goldberg, S. C., *J. Biol. Chem.*, **155,** 591 (1944).
15. Lyman, C. M., Kuiken, K. A., Blotter, L., and Hale, F., *J. Biol. Chem.*, **157,** 395 (1945).
16. Lewis, J. C., and Olcott, H. S., *J. Biol. Chem.*, **157,** 265 (1945).
17. Williams, R. J., McAlister, E. D., and Roehm, R. R., *J. Biol. Chem.*, **83,** 315 (1929).
18. Vickery, H. B., Pucher, G. W., Clark, H. E., Chibnall, A. C., and Westall, R. G., *Biochem. J.*, **29,** 2710 (1935).
19. Wilson, H., and Cannan, R. K., *J. Biol. Chem.*, **119,** 309 (1937).
20. Dakin, H. D., *Z. physiol. Chem.*, **130,** 159 (1923).

21. Chibnall, A. C., and Bailey, K., cited by Cohn, E. J., and Edsall, J. T., Proteins, amino acids and peptides, American Chemical Society monograph series, New York, 358 (1943).
22. Pollack, M. A., and Lindner, M., *J. Biol. Chem.*, **143**, 655 (1942).
23. Niven, C. F., Jr., *J. Bact.*, **47**, 343 (1944).
24. McIlwain, H., Fildes, P., Gladstone, G. P., and Knight, B. C. J. G., *Biochem. J.*, **33**, 223 (1939).
25. McIlwain, H., *Biochem. J.*, **33**, 1942 (1939).
26. Pollack, M. A., and Lindner, M., *J. Biol. Chem.*, **147**, 183 (1943).
27. Wright, L. D., and Skeggs, H. R., *J. Bact.*, **48**, 117 (1944).

THE MICROBIOLOGICAL DETERMINATION OF AMINO ACIDS*

III. ASSAY OF ASPARTIC ACID WITH LEUCONOSTOC MESENTEROIDES

By LUCILE R. HAC AND ESMOND E. SNELL

(*From the Amino Products Division of the International Minerals and Chemical Corporation, Toledo, Ohio, and The University of Texas, Biochemical Institute, and the Clayton Foundation for Research, Austin*)

(Received for publication, February 27, 1945)

Chemical methods for the determination of aspartic acid are similar to those used for glutamic acid. The most accurate values recorded for pure proteins are those of Rittenberg and Foster (2), Chibnall and coworkers (3, 4), Cannan (5), and Kibrick (6). Comparable values have been obtained by the microbiological method of assay here described.

EXPERIMENTAL

Leuconostoc mesenteroides P-60 was the microorganism used for assay.[1] Stock cultures were maintained and the inoculum prepared as previously described. The complete basal medium given in the preceding paper (1) was used. Aspartic acid was omitted, the phosphate content of the medium was increased 4-fold, and the final pH of the medium was adjusted to 6.8. Pure $l(+)$-aspartic acid was used for the standard curve and the assay range was 0.001 to 0.04 mg. per 2.5 ml. of medium. The protein hydrolysates were the same as those used in the assay of glutamic acid. Each sample was analyzed in duplicate at three or four levels of concentration. Values calculated for the different levels were averaged to give the final assay results. Turbidity measurements were used exclusively. The results are given in Fig. 1 and Tables I and II.

DISCUSSION

Standard Curve—A typical standard growth curve for $l(+)$-aspartic acid is plotted in Fig. 1. Growth is proportional to the amount of amino acid present in the medium and the curve is similar to those reported for all other amino acids thus far assayed (10–17) with the exception of glutamic acid (1, 18, 19). The unusual shape of the growth curve for glutamic acid appears to be due to the fact that glutamic acid is not utilized directly but is instead converted to its amide, glutamine, before assimilation (1).

Asparagine can substitute for aspartic acid in a fashion entirely analogous

* For Paper II of this series, see Hac, Snell, and Williams (1).

[1] The amino acid requirements of this organism have recently been described by Dunn *et al.* (7).

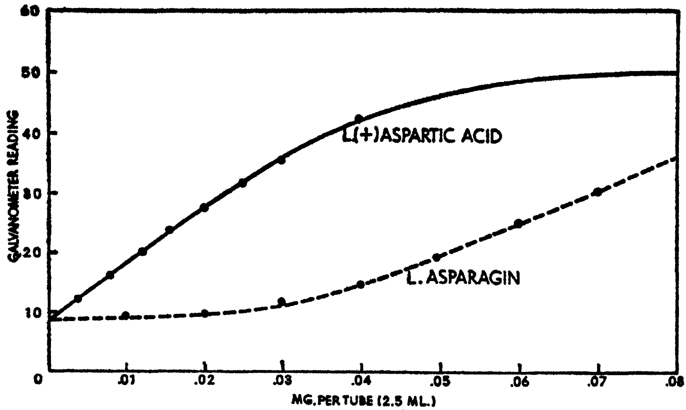


FIG. 1. Comparative response of *Leuconostoc mesenteroides* to aspartic acid and asparagine.

TABLE I

l(+)-*Aspartic Acid Content of Various Pure Proteins**

Assay No.	Labco casein	Lacto-globulin†	Egg albumin†	Gliadin‡	Horse hemo-globin (recrystal-lized 3 times)†	Horse car-boxyhemo-globin (recrystal-lized 2 times)§	Gelatin (Knox)	Silk fi-broin†
I	6.92	11.23	9.0	2.97	9.82	10.20	5.91	2.10
II	6.83	11.00	8.80	3.10	9.55	10.34	5.44	3.25
III	6.81	11.20	7.35‖	3.00	9.62		5.32	2.70
IV	7.03	12.00	8.80	3.00	9.50		5.75	2.58
V	7.40	10.33	11.10‖					
VI	7.30	11.65	9.20					
VII	7.10	10.50	8.30					
VIII	6.91	12.20						
Average.....	7.0	11.3	8.9	3.0	9.6	10.3	5.6	2.7
Corrected value¶....	7.2	11.5	9.3	3.3	10.3	10.8	6.8	2.8
Values reported in literature.	6.68(3)	11.1** 9.88(4)	8.13(4)	1.40(3)	8.9(8)	10.5§ 6.75(3)	3.4(9)	

* Each value is the average of duplicate determinations at three or four levels of concentration.

† Protein furnished by the late Dr. Max Bergmann.

‡ Protein furnished by Dr. H. S. Olcott.

§ Protein furnished by Dr. G. L. Foster. Analysis by isotope dilution, 10.5 per cent aspartic acid.

‖ Value omitted from the average.

¶ Corrected for moisture content. We are indebted to Beverly M. Guirard for the moisture determinations.

** Analysis by isotope dilution. Personal communication from Dr. G. L. Foster.

to glutamic acid for glutamine, and its growth curve (Fig. 1) is similar to that of glutamic acid. It was shown previously that the addition of ammonium sulfate to the basal medium for glutamic acid assay furthered ammonia transfer and the conversion to glutamine. In the case of aspartic acid, the ammonium ion inhibited growth slightly. Additional phosphate eliminated this inhibitory effect with aspartic acid, but delayed growth in the medium for glutamic acid.

The utilization of glutamic and aspartic acids is in direct contrast to each other. Whereas glutamic acid is less readily available for growth than glutamine, asparagine is less readily available than aspartic acid.[2] This is interpreted to mean that glutamic acid is converted to glutamine before assimilation, whereas asparagine must be converted to free aspartic acid

TABLE II

Recovery of l(+)-Aspartic Acid Added to Protein Hydrolysates

Protein	l(+)-Aspartic acid			
	Found per aliquot	Added	Total found	Per cent recovery
	mg.	*mg.*	*mg.*	
Casein	0.0067	0.002	0.0085	99.7
	0.0067	0.004	0.0108	100.9
	0.0099	0.002	0.0120	100.8
	0.0099	0.006	0.0163	102.5
	0.0125	0.002	0.0147	101.4
	0.0125	0.006	0.0190	102.7
Lactoglobulin	0.0106	0.002	0.0130	103.1
	0.0106	0.006	0.0166	100.0
	0.0220	0.002	0.0248	103.3
	0.0220	0.006	0.0262	90.4
Horse carboxyhemoglobin	0.0144	0.008	0.0222	99.1
	0.0180	0.010	0.0288	102.8

Asparagine must play a dual rôle in bacterial metabolism since it is necessary for rapid growth of some microorganisms in media which contain aspartic acid (20, 21).

Reliability of Assay—The results of assay of several pure proteins are listed in Table I. Consistent values were obtained at various levels of concentration and on repeated assay. The values are somewhat higher than those of Chibnall *et al.*, obtained by an isolation procedure (3, 4), but he has indicated that certain of his values may still be low. Analyses by isotope

[2] The relative inactivity of *l*-asparagine for this organism as compared with *l*(+)-aspartic acid has been independently noted by E. Brand, L. J. Saidel, and W. H. Goldwater (private communication from Dr. Brand).

dilution have also given higher results[3] and the assay of two proteins for which these data were available is in excellent agreement with that method.

Recovery of aspartic acid added to protein hydrolysates (Table II) was with but one exception within 4 per cent of theoretical.

SUMMARY

Leuconostoc mesenteroides P-60 has been used successfully for the quantitative determination of $l(+)$-aspartic acid in protein hydrolysates. The following values were obtained with the pure proteins studied: Labco casein 7.2 per cent, lactoglobulin 11.5 per cent, egg albumin 9.3 per cent, gliadin 3.3 per cent, horse hemoglobin 10.3 per cent, horse carboxyhemoglobin 10.8 per cent, gelatin 6.8 per cent, and silk fibroin 2.8 per cent. Asparagine in high concentrations can substitute for aspartic acid. Aspartic acid appears to be utilized directly as contrasted with its homologue, glutamic acid, which is probably converted into the amide before assimilation (1).

BIBLIOGRAPHY

1. Hac, L. R., Snell, E. E., and Williams, R. J., *J. Biol. Chem.*, **159**, 273 (1945).
2. Rittenberg, D., and Foster, G. L., *J. Biol. Chem.*, **133**, 737 (1940).
3. Bailey, K., Chibnall, A. C., Rees, M. W., and Williams, E. F., *Biochem. J.*, **37**, 360 (1943).
4. Chibnall, A. C., Rees, M. W., and Williams, E. F., *Biochem. J.*, **37**, 372 (1943).
5. Cannan, R. K., *J. Biol. Chem.*, **152**, 401 (1944).
6. Kibrick, A. C., *J. Biol. Chem.*, **152**, 411 (1944).
7. Dunn, M. S., Shankman, S., Camien, M. N., Frankl, W., and Rockland, L. B., *J. Biol. Chem.*, **156**, 703 (1944).
8. Chibnall, A. C., and Bailey, K., cited by Cohn, E. J., and Edsall, J. T., Proteins, amino acids and peptides, American Chemical Society monograph series, New York, 358 (1943).
9. Dakin, H. D., *J. Biol. Chem.*, **44**, 499 (1920).
10. Shankman, S., *J. Biol. Chem.*, **150**, 305 (1943). Shankman, S., Dunn, M. S., and Rubin, L. B., *J. Biol. Chem.*, **151**, 511 (1943).
11. Kuiken, K. A., Norman, W. H., Lyman, C. M., Hale, F., and Blotter, L., *J. Biol. Chem.*, **151**, 615 (1943).
12. McMahan, J. R., and Snell, E. E., *J. Biol. Chem.*, **152**, 83 (1944).
13. Greene, R. D., and Black, A., *J. Biol. Chem.*, **155**, 1 (1944).
14. Hegsted, D. M., *J. Biol. Chem.*, **152**, 193 (1944).
15. Baumgarten, W., Garey, J. C., Olsen, M. J., Stone, L., and Boruff, C. S., *J. Am. Chem. Soc.*, **66**, 1607 (1944).
16. Schweigert, B. S., McIntire, J. M., Elvehjem, C. A., and Strong, F. M., *J. Biol. Chem.*, **155**, 183 (1944).
17. Dunn, M. S., Camien, M. N., Shankman, S., Frankl, W., and Rockland, L. B., *J. Biol. Chem.*, **156**, 715 (1944).
18. Lewis, J. C., and Olcott, H. S., *J. Biol. Chem.*, **157**, 265 (1945).
19. Lyman, C. M., Kuiken, K. A., Blotter, L., and Hale, F., *J. Biol. Chem.*, **157**, 395 (1945).
20. Niven, C. F., Jr., *J. Bact.*, **47**, 343 (1944).
21. Feeney, R. E., and Strong, F. M., *J. Am. Chem. Soc.*, **64**, 881 (1942).

[3] G. L. Foster, personal communication.

CITRIC ACID FORMATION FROM ACETOACETIC AND OXALACETIC ACIDS*

By F. EDMUND HUNTER and LUIS F. LELOIR

(*From the Department of Pharmacology, Washington University School of Medicine, St. Louis*)

(Received for publication, April 9, 1945)

Breusch (1) reported the discovery of an enzyme, citrogenase, which catalyzes the formation of citric acid from oxalacetic acid and β-keto fatty acids, including acetoacetic acid. According to the proposed mechanism of reaction the β-keto acid and oxalacetic acid would condense to form an intermediate compound which would undergo hydrolysis to yield citric acid and a fatty acid shortened by the loss of 2 carbon atoms. The 2-carbon group removed from the fatty acid would appear in the citric acid and be metabolized to carbon dioxide and water by way of the "citric acid" or tricarboxylic acid cycle. Oxalacetic acid would be regenerated and the process could be repeated as often as β oxidation of the fatty acid occurred.

Independently Wieland and Rosenthal (2) published findings similar to those of Breusch with respect to acetoacetic acid, and Martius (3) has added partial confirmation of these reports. However, Weil-Malherbe (4) and Krebs and Eggleston (5) have reported that they found no evidence for the existence of a citrogenase for metabolizing acetoacetic acid. In the present paper the observations concerning citric acid formation from acetoacetic and oxalacetic acids by kidney tissue have been confirmed and the nature of this enzymatic reaction has been studied. Since the completion of this work Buchanan *et al.* (6) have reported that heavy carbon in acetoacetic acid added to homogenized kidney cortex appears in α-keto glutaric and fumaric acids, members of the tricarboxylic acid cycle.

Methods

Preparations—The following methods were used: oxalacetic acid, Krampitz and Werkman (7); acetoacetic acid, Ljunggren (8); acetyl phosphate, Lipmann and Tuttle (9); sodium pyruvate, Robertson (10).

Ketone Body Estimation—Acetoacetic acid was determined by the aniline method (Edson (11)) or by the method of Weichselbaum and Somogyi (12). In a number of experiments both methods were used. For the determination of acetoacetic acid by the aniline method in the presence of oxalacetic acid, use was made of the effect of cupric ions on the decomposition of the latter acid (Krebs (13)). The contents of the Warburg flasks (2 to 3 ml.)

* This work was supported in part by a grant from the Rockefeller Foundation.

were acidified with 0.3 ml. of glacial acetic acid and 0.3 ml. of 20 per cent copper sulfate was added. The oxalacetic acid was completely decarboxylated in 20 to 30 minutes at 25°, while the acetoacetic acid remained unaffected. Then 0.4 ml. of 40 per cent aniline in glacial acetic acid was tipped in from a side bulb in order to decarboxylate the acetoacetic acid.

β-Hydroxybutyric acid was determined according to the method of Weichselbaum and Somogyi (12).

Citric Acid Estimation—A modification of the pentabromoacetone method of Pucher, Sherman, and Vickery (14) was used. The preparation of the samples and the oxidation with permanganate were carried out as described by these authors. Then the mixture was decolorized with a slight excess of freshly prepared saturated sodium nitrite solution (about 1 ml.). 25 ml. of petroleum ether (Mallinckrodt, b.p. 35–60°) were added and the separatory funnel shaken vigorously for 1 minute. After complete separation of the petroleum ether the aqueous layer was discarded. The petroleum ether was washed twice with 10 ml. of water. Shaking for 10 seconds was sufficient. After the second wash water had been drained off, 5 ml. of 2.5 per cent sodium sulfide in 40 per cent ethylene glycol were added to the petroleum ether and the funnel was shaken about 15 seconds. All wash water was shaken out of the stem of the funnel. The yellow-colored layer was drained into a colorimeter tube graduated at 10 ml. The extraction was repeated twice with 2 ml. portions of the sulfide reagent. The extracts were combined in the colorimeter tube, made up to 10 ml. with the sulfide reagent, mixed, centrifuged for a short period, and read in a Klett-Summerson photoelectric colorimeter with Filter 42. At least one standard citric acid was run simultaneously with each group of determinations.

The described procedure has a number of advantages, with essentially the same accuracy as the original method. A great deal of time is saved. The use of sodium nitrite instead of ferrous sulfate for destroying excess permanganate and bromine does not interfere with the method and eliminates much of the tedious washing of the petroleum ether layer to get rid of the iron.

One petroleum ether extraction of the pentabromoacetone seems sufficient; determinations carried out with a second petroleum ether extract alone gave values equivalent to water blanks. Goldberg and Bernheim (15) have recently reported this same observation. With only one extraction fewer separatory funnels and manipulations are required.

Ethylene glycol proved to be a better color stabilizer than pyridine. It has the additional advantage that it is immiscible with petroleum ether and therefore can be added during the extraction. The use of ethylene glycol in this manner facilitates making the time between the development of the color and the measurement with the colorimeter equal for all samples. A

linear relationship between color and citric acid was found between 0.2 and 1.0 mg. of citric acid, but not with smaller or larger amounts.

Enzyme Preparations—All experiments reported in this paper were carried out with enzyme preparations from kidney cortex, but the presence of the enzyme in heart muscle has also been confirmed. Breusch (1) states that he extracted citrogenase from tissue with 0.5 per cent sodium bicarbonate, while other workers have used slices, homogenates, or minced tissue. Preliminary experiments were carried out either with homogenates or with the cloudy extracts obtained from homogenates by centrifugation at 2500 R.P.M. for 10 to 15 minutes. In both cases 2 volumes of 0.5 per cent sodium bicarbonate were used with 1 volume of tissue. These preparations showed some activity. Experiments with the multispeed attachment for International centrifuges revealed that the enzyme was not truly extracted by sodium bicarbonate, but was associated with particles which were separated by high speed centrifugation (see Table I). The procedure finally adopted for preparing the enzyme system is described in the next paragraph.

The kidneys were removed from dogs anesthetized with ether, iced, and the cortex homogenized at 0° with 1 volume of distilled water in a stainless steel homogenizer of the type described by Potter and Elvehjem (16). The homogenate was strained through muslin and 0.05 volume of 10 per cent potassium chloride solution added with mixing. This preparation was centrifuged in the cold room for 15 minutes in the multispeed attachment of an International centrifuge at about 12,000 R.P.M. The supernatant fluid was discarded and the precipitate suspended in distilled water sufficient to restore the original volume. 10 per cent potassium chloride solution (one-tenth of the volume of the water) was added and the mixture thoroughly stirred. The tubes were again centrifuged at high speed. This washing of the tissue particles was repeated once more. Finally the precipitate was thoroughly suspended in enough 0.8 per cent sodium chloride to make about one-third of the original volume of homogenate. This yielded a thin yellowish paste which could be pipetted easily. All steps of the preparation were carried out in the cold. In a few cases kidney tissue from cats and rabbits was used, but attempts to use pig kidney obtained from the slaughter-house were abandoned because the material was inactive in about half of the trials. Acetone powders were completely inactive.

Experimental Procedure—Experiments were carried out in 25 ml. or 50 ml. Erlenmeyer flasks when citric acid only was estimated. Borate or bicarbonate buffer at pH 7.4 was used and all solutions were adjusted to this pH. Unless otherwise indicated 0.5 ml. of enzyme preparation was used and the final volume was made up to 1.1 ml. by various additions or by water. After the air above the liquid had been replaced with the appropriate gas, the flasks were stoppered and shaken horizontally during

the incubation period. Trichloroacetic acid filtrates were prepared for citric acid determination.

The balance experiments were carried out in Warburg flasks with 1 ml. of enzyme preparation and a final volume of 2.2 to 2.5 ml. Borate buffer was used when oxygen uptake was measured. For anaerobic experiments, bicarbonate buffer was used and the flasks were filled with nitrogen containing 5 per cent carbon dioxide. The gas evolution under these conditions is referred to as acid formation. At the end of the incubation period the contents of the flask were diluted with a measured amount of distilled water and an aliquot removed for estimation of ketone bodies according to the method of Weichselbaum and Somogyi. Acetoacetic acid estimation by the aniline method was carried out directly on the material remaining in the flask, without the removal of proteins. Then 5 ml. of 10 per cent trichloroacetic acid were added to the flask with thorough mixing. The filtrate was used for citric acid determinations. Aniline does not interfere in the citric acid estimation provided sufficient bromine is added and the precipitate formed is removed.

Results

Stability of Enzyme System—Whole kidney tissue, kidney homogenate, and the preparation of washed tissue particles lose little activity during several hours at 0°. Homogenates and washed preparations aged 16 to 20 hours at 0° still possessed about 40 per cent of the original activity. Dialysis of homogenates for 16 to 20 hours at 0° resulted in loss of most of the activity. In about half of these dialyzed preparations some activity could be restored by adding a boiled muscle or kidney extract or glutathione.

Additions Necessary for Citrate Formation—Addition of oxalacetate to homogenates caused some citrate formation. When acetoacetate was also added, there was a considerable increase in citrate formation, but when the tissue particles were washed several times, as described in the procedure for preparing the enzyme, the addition of acetoacetate caused little increase in citrate formation over that with oxalacetate alone. Some 50 to 60 per cent of the original extra citrate formation caused by acetoacetate could be restored by adding to the tissue particles the supernatant fluid from the first centrifugation. Heating the first supernatant fluid to 80° for 5 minutes did not destroy its ability to restore the activity of the system (see Table I).

These experiments indicated that some factor necessary for citrate formation from acetoacetate was removed by washing of the tissue particles. This factor could be restored by addition of a boiled extract prepared from kidney or heart, or a water extract of dried yeast, but was not present in the ash. In the course of the search for the unknown factor the following substances were tested and found to be without effect: adenosine triphos-

phate, adenylic acid, cozymase, cocarboxylase, cytochrome c, cysteine, riboflavin, glycine, alanine, and pantothenic, ascorbic, p-aminobenzoic, succinic, fumaric, aspartic, and pyruvic acids.

The only substances found which could replace the heated tissue extract in restoring the activity of the system were glutathione, glutamic acid, and α-ketoglutaric acid. The effect of glutathione was discovered first and for a time it was believed to be the necessary substance. However, later experiments revealed that glutamic acid and α-ketoglutaric acid were more effective than glutathione and were able to restore activity to some preparations which were not affected by glutathione. This led to the conclusion that either glutamic acid or α-ketoglutaric acid was the essential substance.

TABLE I

Citric Acid Formation in Homogenates, Extracts, and Washed Particles Prepared from Kidney Tissue

Dog kidney cortex homogenized at 0° in 1.5 volumes of 0.5 per cent sodium bicarbonate; part centrifuged at 12,000 R.P.M. for 15 minutes in the cold room; precipitate resuspended in the original volume of bicarbonate solution or in its supernatant fluid; 3 ml. of tissue preparation + 100 micromoles of oxalacetate + 40 micromoles of acetoacetate; barium chloride 0.02 M; final volume 4.2 ml.; nitrogen with 5 per cent carbon dioxide as gas; pH 7.5; 1 hour at 37°. The results are given in micromoles.

Experiment No.	Tissue preparation	Citrate formed		
		With oxalacetate alone	With acetoacetate alone	With oxalacetate + acetoacetate
1	Homogenate	14.1	8.3	33
	Supernatant fluid	0.9	0.6	1.8
	Ppt. resuspended in bicarbonate	13.8	1.5	24
2	Homogenate	2.6		23
	Ppt. (washed 2 times)	1.4		2.8
	" + first supernatant fluid	3.6		15.9
	" + heated first supernatant fluid	2.4		13.8

These two substances seemed equally effective in equimolecular concentrations. The effect of glutathione may be due to glutamic acid released by hydrolysis (17). Table II gives the results of some of the experiments with different additions. The data clearly indicate that both oxalacetate and α-ketoglutarate (or glutamate) must be present if the addition of acetoacetate is to cause a large increase in citrate formation. As a result of the work of Buchanan *et al.* (6) it seems safe to assume that this represents a real conversion of acetoacetate to citrate.

Specificity of Oxalacetate As Substrate—Oxalacetate could not be replaced by pyruvate, fumarate, malate, or succinate in anaerobic experiments (Table III). Aerobically with added cytochrome c malate was slightly

active. Evidently it is converted to oxalacetate slowly by these preparations. In intact tissue any substance which can be converted to oxalacetate should be able to replace this substance.

TABLE II

Effect of Various Substances on Citrate Formation

Experiments with washed tissue particles suspended in 0.025 M sodium bicarbonate; 0.02 M barium chloride present; 50 micromoles of oxalacetate, 8 to 15 micromoles of acetoacetate, 10 micromoles of additions unless otherwise indicated; total volume 1.1 ml.; gas, 5 per cent carbon dioxide in oxygen; 60 to 90 minutes at 28°. The results are given in micromoles.

Experiment No.	Additions	Citrate formed		
		With oxalacetate alone	With oxalacetate + acetoacetate	With acetoacetate alone
1	None	5.3	9.2	
	l-Glutamate	4.6	20.6	
	α-Ketoglutarate	4.0	19.2	
	Glutathione	4.4	11 7	
2	None	5.9	9.2	
	Glutathione, 5 micromoles	4.4	14.4	
	l-Glutamate	5.5	21.6	
	Glycine	5.7	8.9	
	l-Cysteine	7.3	11.2	
3	None	5.6	7.3	
	Glutathione	4.4	15.1	
	l-Cysteine	3.6	4.9	
4	None	5.4	8.6	
	l-Glutamate	3.9	13.4	1 4
	α-Ketoglutarate	3.3	10.4	1.3
	Glutathione	3.7	6.9	
	l-Aspartate	6 4	7 3	
	Succinate	3.9	5.2	
	dl-Alanine	4.6	5.8	
5	None	4.1	7.6	
	l-Glutamate, 2 5 micromoles	3.2	10 0	
	" 10 "	2.4	12 1	1.5
	" 20 "	2.2	8.4	
	α-Ketoglutarate, 2.5 micromoles	2.8	9.2	
	" 10 "	2.3	11.2	3.1
	" 20 "	2.1	10.5	

Specificity of Acetoacetate As Substrate—As may be seen from Table IV, some increases in citrate formation were found with β-hydroxybutyrate, crotonate, and butyrate, but the effect was smaller than with acetoacetate and these substances are known to be transformed into acetoacetate. Py-

ruvate gave some increase. Changes caused by acetate were probably not significant. Acetyl phosphate in different amounts was tested in several experiments, with and without glutamate, with negative results. Of the group of substances tested, acetoacetate is probably the only one which can serve as substrate. Whether the enzyme also acts on other β-keto fatty acids, as reported by Breusch, has not been determined.

Balance between Citrate Formation, Acid Formation, and Acetoacetate Disappearance—Tables V and VI show the results of experiments designed to determine the relation between acetoacetate disappearance and citrate formation.

The addition of oxalacetate to the kidney preparation results in the for-

TABLE III

Specificity of Oxalacetate

Experiments with washed tissue particles in 0.025 M sodium bicarbonate; 0.02 M barium chloride present; 10 micromoles of glutamate present in all samples; total volume 1.1 ml.; gas, 5 per cent carbon dioxide in nitrogen; 60 minutes at 28°. The values are given in micromoles.

Experiment No.	Additions		Citrate formed	
			Without acetoacetate	With acetoacetate (9 micromoles)
1	Oxalacetate	50	4.0	13.0
	Fumarate	30	0.6	3.1
	l-Malate	30	1.8	4.4
	Succinate	30	1.3	1.4
	Pyruvate	30	0.7	1.9
2	None		0.8	1.4
	Oxalacetate	50	3.9	13.4
	Fumarate	100	1.2	2.4
	Pyruvate	75	1.1	2.1

mation of some citrate anaerobically as well as aerobically. When α-ketoglutarate or glutamate is added, the amount of citrate formed from oxalacetate is decreased. Martius (3) found that α-ketobutyrate decreased citrate formation from oxalacetate and pyruvate in heart muscle. The mechanism is probably the same in the two cases. These α-keto acids probably produce a competitive inhibition of citrate formation from pyruvate. In the present experiments pyruvate would result from decarboxylation of part of the oxalacetate. Although glutamate and α-ketoglutarate decrease the citrate formation with oxalacetate alone, they greatly increase the extra citrate formed upon the addition of acetoacetate. The differences in the amount of citrate found cannot be explained on the basis of

changes in citrate oxidation, for very little citrate was oxidized by the preparations used (Table VII).

Anaerobic Balances—The disappearance of acetoacetate (over and above that which is reduced to β-hydroxybutyrate) is small with oxalacetate alone, or with glutamate or α-ketoglutarate alone, and attains its maximum only when both oxalacetate and α-ketoglutarate or glutamate are present. As

TABLE IV

Specificity of Acetoacetate

Experiments with washed tissue particles in 0.025 M sodium bicarbonate; 0.02 M barium chloride present; 50 micromoles of oxalacetate, 5 micromoles of glutathione or 10 micromoles of glutamate, and 10 micromoles of additions; total volume 1.1 ml.; gas, 5 per cent carbon dioxide in nitrogen; 60 to 90 minutes at 28°. The results are given in micromoles.

	Experiment No.	Additions	Citrate formed
Glutathione	1	None	4.7
		Acetoacetate	18.3
		Acetate	7.3
		Acetone	4.7
		Ethanol	4.6
		dl-β-Hydroxybutyrate	11.5
		Pyruvate	6.2
		Crotonate	7.2
		Butyrate	7.5
		Octanoate	5.3
Glutamate	2	None	5.6
		Acetoacetate	14.7
		Acetaldehyde	5.3
		Acetate	5.3
	3	None	6.7
		Acetoacetate	18.4
		Acetate	7.1
		Pyruvate	9.8
	4	None	4.0
		Acetoacetate	17.6
		Acetyl phosphate	4.9
		Pyruvate	5.0

nearly as could be determined, when acetoacetate was added to the otherwise complete system, 2 molecules of extra citrate[1] were formed for each

[1] According to Krebs and Eggleston (18) the amount of isocitrate and *cis*-aconitate in enzymatic equilibrium with the citrate is about 10 per cent, or much lower, 4 per cent, in the presence of magnesium ions. The citrate values reported have not been corrected for isocitrate and *cis*-aconitate, but this error is small, for with the washed kidney preparation and 0.02 M barium chloride only 8 per cent of added citrate disappeared aerobically. This figure represents the amount oxidized plus the amount converted into isocitrate and *cis*-aconitate.

molecule of acetoacetate which disappeared. For example, in Experiment 1 of Table V, 13.5 micromoles of citrate were formed with oxalacetate plus α-ketoglutarate. When acetoacetate also was added, completing the system, 30.2 micromoles of citrate were formed. Thus the extra citrate due to the addition of acetoacetate was 16.7 micromoles. The amount of citrate formed from oxalacetate alone is not used as a control value because

TABLE V

Anaerobic Balance

Warburg vessels containing 0.2 ml. of 0.4 M sodium bicarbonate + 0.1 ml. of 0.45 M barium chloride + 1.0 ml. of a suspension of washed tissue particles; 50 minutes at 30°; pH 7.3; gas, 5 per cent carbon dioxide in nitrogen; total volume 2.4 ml. All values are given in micromoles.

Experiment No.	Additions			Ketone body recovery		Ketone body disappearance	Citrate formed	Acid formed
	Oxalacetate	Acetoacetate	α-Ketoglutarate	Acetoacetate	β-Hydroxybutyrate			
1	100			0.3	0.3		19.6	
	100	20.5		15.0	3.1	2.4	23.5	
	100	20.5	20	5.4	5.7	9.4	30.2	
		20.5	20	10.1	6.9	3.5	1.4	
		20.5		14.8	5.0	0.7	0.9	
	100		20	1.6	0.5		13.5	
2	100			0.2	0.6		25.1	26
	100	14		8.3	4.3	1.4	31.5	28
	100	14	20	1.0	2.7	10.3	36	33
		14	20	4.9	7.2	1.9	1 5	11.9
		14		9.9	4.0	0.1	2.0	3.7
	100		20	0.5	0.4		17.7	31
3	100			0.5	*		12.6	12.3
	100	16		14.3		1.7	16.0	15.5
	100	16	20	6.9		9.1	22	23
		16	20	12.4		3.6	3.3	5.0
		16		16.0		0	3.0	1.5
	100		20	0.3			6.3	18
			20	0.3			4.4	1.8
				1.0			2.7	0.6

* β-Hydroxybutyrate was not determined in Experiment 3.

α-ketoglutarate decreases this figure and this effect of α-ketoglutarate must still be present, at least in part, in the complete system. The ketone body disappearance in the complete system was 9.4 micromoles, as against 0.7 for the control with acetoacetate alone. Thus completion of the system increased the ketone body disappearance by 8.7 and the citrate formation by 16.7 micromoles. Small amounts of ketone bodies disappeared with

oxalacetate plus acetoacetate and with acetoacetate plus α-ketoglutarate. These changes were very probably due to the fact that small amounts of the substance necessary to complete the system were still present in the tissue

TABLE VI

Aerobic Balance

Warburg vessels containing 0.2 ml. of 0.5 M borate buffer of pH 7.5 + 0.5 mg. of cytochrome c preparation + 0.1 ml. of 0.45 M barium chloride + 1 ml. of a suspension of washed tissue particles; gas, oxygen; total volume 2.4 ml. Experiments 1 and 3, 60 minutes at 30°, Experiment 2, 100 minutes at 25°. All values are given in micromoles.

Experiment No.	Additions			Ketone body recovery		Ketone body disappearance	Citrate formed	Oxygen consumed
	Oxalacetate	Acetoacetate	α-Ketoglutarate	Acetoacetate	β-Hydroxybutyrate			
1	100			0.5	0.7		25	5.2
	100	16.5		12.9	0.7	2.9	30 3	7.1
	100	16.5	20	5.4	0.9	10.2	41	15.8
		16.5	20	3.6	2.0	10.9	13.5	25.2
		16.5		13.7	0.7	2.1	3.5	4.3
	100		20	0.5	0.4		14.3	7.5
2	100			0.4	*		23	11.1
	100	15		13.8		1.2	33	12 0
	100	15	20	5.7		9.3	39	22
		15	20	6.3		8.7	15.5	34
		15		14.3		0 7	10.9	11.8
	100		20	0.6			14.6	12.7
			20	0.3			5.4	26
				0			5 1	9.0
3†	100			1.5	*		22	6.3
	100	16		14.5		1 5	28	6.3
	100	16	20	2.8		13.2	46	23
		16	20	10.2		5.8	13	19.3
		16		15.0		1 0	5.1	4.3
	100		20	1.3			16	11.0
			20	0			6.0	18.1
				2.2			4.8	4.2

* β-Hydroxybutyrate was not determined in Experiments 2 and 3.

† With glutamate instead of α-ketoglutarate.

particles or were formed during the experiment. The data are in agreement with the equation proposed by Wieland and Rosenthal (2).

(1) 2 oxalacetate + 1 acetoacetate → 2 citrate

Additional information concerning the reactions taking place is furnished by the acid formation, as measured by carbon dioxide evolution from the

bicarbonate buffer. The acid formation by the system with oxalacetate alone represents approximately 1 molecule of acid per molecule of citrate formed. (The decarboxylation of oxalacetate to pyruvate, which takes place rapidly in these experiments, occurs without any acid formation.) This relationship is what would be expected on the basis of either of the following equations proposed by Krebs *et al.* (19), in which one extra acid group is produced for each citrate formed.

(2) 3 oxalacetate → 1 citrate + 1 malate + 2 bicarbonate

(3) 2 oxalacetate + 1 pyruvate → 1 citrate + 1 malate + 1 bicarbonate

When glutamate or α-ketoglutarate is added to the washed tissue plus oxalacetate, there is a large acid formation not accounted for by the citrate

TABLE VII

Citric Acid Oxidation by Washed Kidney Tissue Particles

1.0 ml. of suspension of washed tissue particles + 0.5 mg. of cytochrome *c* preparation + 0.2 ml. of 0.5 M borate buffer at pH 7.4 + 1.2 ml. of total additions; 0.02 M NaCl or $BaCl_2$, 0.01 M phosphate, 0.004 M $MgCl_2$, 1 mg. of adenylic acid, and 18.5 micromoles of citrate when indicated; gas, oxygen; 60 minutes at 30°. All values are given in micromoles.

Additions to tissue	Oxygen consumption	Citrate after incubation
NaCl	0.8	0.6
" + citrate	1.4	17.3
$BaCl_2$	1.0	0.8
" + citrate	2.4	17.8
NaCl + phosphate, $MgCl_2$, adenylic acid	1.4	1.0
" + " " " " + citrate	1.6	18.5
$BaCl_2$ + " " " "	2.1	0.7
" + " " " " + citrate	3.6	17.7

formed. This is most probably the result of oxidation of α-ketoglutarate to succinate by the oxalacetate.

(4) 1 α-ketoglutarate + 1 oxalacetate → 1 malate + 1 succinate + 1 bicarbonate

Glutamate, α-ketoglutarate, and acetoacetate, when added separately, cause very little acid formation. When acetoacetate is added along with α-ketoglutarate, there is some acid formation, indicating that acetoacetate can oxidize α-ketoglutarate to succinate.

The acid formation, acetoacetate disappearance, and extra citrate formation of the complete system are in general agreement with the following equation, in which the acid formation to citrate formation ratio is 1 and the

citrate formation to ketone body disappearance ratio is 2. Equation 5 represents the sum of Equations 1 and 4.

(5) 3 oxalacetate + 1 acetoacetate + 1 α-ketoglutarate $\rightarrow$ 2 citrate + 1 malate
+ 1 succinate + 1 bicarbonate

Aerobic Balances—Cytochrome *c* was added in the experiments presented in Table VI. Glutamate and α-ketoglutarate are the only substances which cause a marked increase in the oxygen uptake of the system. When oxalacetate is also present, the oxygen uptake is lower, probably because oxalacetate can replace oxygen as an oxidant.

Aerobically the relation of acetoacetate disappearance to citrate formation in the complete system is the same as in the anaerobic experiments, but with α-ketoglutarate alone large amounts of acetoacetate disappear, yet do not appear as β-hydroxybutyrate or as citrate. This fact suggests that the primary reaction is the conversion of acetoacetate to an intermediate compound which condenses with oxalacetate, if present, to form citrate.

Influence of Oxygen—Wieland and Rosenthal (2) reported that oxygen was necessary for citrate formation from oxalacetate and acetoacetate. Weil-Malherbe (4) found no citrate formation from acetoacetate with tissue slices anaerobically. The fact that the reaction occurred anaerobically in our experiments is probably due to the excess oxalacetate used, which acted as an oxidant. In some of our experiments with small amounts of oxalacetate and crude homogenates very little citrate was formed anaerobically. These results can be explained by the fact that a large amount of oxalacetate is removed by reduction and by decarboxylation, for we have found that if enough oxalacetate is added the reaction proceeds well anaerobically.

Aerobically, considerable amounts of acetoacetate disappear in all samples which have α-ketoglutarate added; *i.e.*, in all samples in which α-ketoglutarate oxidation is taking place. The disappearance may not always equal that in the complete system, but it is much greater than the controls with acetoacetate alone or with acetoacetate plus oxalacetate. Anaerobically, acetoacetate disappears (over and above that which is reduced) only when oxalacetate and α-ketoglutarate are both present and not with addition of α-ketoglutarate alone. This is because anaerobically α-ketoglutarate is oxidized to a significant extent only in the samples containing the excess oxalacetate. It seems clear that the removal of acetoacetate is dependent on α-ketoglutarate oxidation. If molecular oxygen is not available, oxalacetate can act as the oxidant in this reaction.

Action of Inhibitors—The extra citrate formation caused by the addition of acetoacetate to the otherwise complete system was not influenced by 0.01 M malonate and cyanide or 0.02 M fluoride. Azide in 0.01 M concentration and 0.001 M iodoacetate produced 20 to 25 per cent inhibition. The

system was completely inhibited by 0.01 M iodoacetate, selenite, and arsenite.

Action of Barium Ions—Barium ions were added in nearly all experiments because Wieland and Rosenthal suggested that they decrease citrate oxidation. However, later experiments indicated that very little citrate was oxidized by the washed kidney preparations with or without barium, as was ascertained by measuring citrate disappearance and oxygen uptake with added cytochrome *c* (Table VII). The barium ions did tend to stimulate acetoacetate disappearance and citrate formation in some of our experiments. Further work will be necessary to determine the mechanism of this effect.

DISCUSSION

Although citrate formation has been followed in most studies of the enzyme citrogenase, it is very probable that an intermediate analogous to *cis*-aconitic acid is the first substance formed from oxalacetic and acetoacetic acids. That *cis*-aconitate or isocitrate and not citrate must be formed first has been demonstrated in the case of pyruvate by Wood *et al.* (20) and indicated in the case of acetoacetate by the work of Buchanan *et al.* (6). *Cis*-aconitate would give rise to isocitrate and the remainder of the tricarboxylic acid cycle would follow from this point (Krebs (21)). The citrate is formed from *cis*-aconitate in a side reaction, especially when the cycle is stopped at isocitrate. Krebs and Eggleston (18) have shown that the equilibrium isocitrate $\rightleftarrows$ *cis*-aconitate $\rightleftarrows$ citrate is such that from 89 to 96 per cent of the material is citrate under different circumstances. For this reason it is possible to use citrate formation to follow what is really *cis*-aconitate formation. Since very little citrate is oxidized by our preparations, the cycle must be blocked at the isocitrate step, presumably because of absence or inactivity of the necessary enzymes.

The experiments show that very little acetoacetate disappears and very little extra citrate is formed when acetoacetate is incubated with washed kidney preparation and oxalacetate. When α-ketoglutarate, glutamate, or glutathione is also added, acetoacetate disappears and extra citrate is formed. Reduction to β-hydroxybutyrate accounts for only a small part of the acetoacetate disappearance.

The increase in oxygen uptake on the addition of α-ketoglutarate indicates that this substance is oxidized by our preparations. Anaerobically in the presence of oxalacetate it produces considerable acid formation which can be explained by oxidative decarboxylation. In this case oxalacetate would be the oxidant. It seems therefore likely that the oxidation of α-ketoglutarate is necessary for the transformation of acetoacetate into citrate in our preparations. The activity of glutamate is probably due to

conversion to α-ketoglutarate by oxidation or by transamination with some of the oxalacetate. Such reactions proceed very rapidly in kidney. Glutathione is less active than glutamate. It is known (Woodward and Reinhart (17)) that glutathione can be split into cysteine, glycine, and glutamic acid by kidney extracts. Further work is necessary to determine whether the oxidation of other substances can replace α-ketoglutarate oxidation. Buchanan *et al.* (6) reported that a number of substances stimulated the disappearance of acetoacetate, but did not determine whether this effect was due to their oxidation or to their conversion to oxalacetate, as was possible with all the substances used.

It appears that citrate can be formed by two mechanisms: (*a*) from oxalacetate and pyruvate, (*b*) from oxalacetate and acetoacetate with the simultaneous oxidation of α-ketoglutarate. Either two entirely different mechanisms operate or a common substance formed from pyruvate and from acetoacetate condenses with oxalacetate to form the precursor of citrate. Krebs (21) has suggested and Martius (3) has supported the idea that a reactive 2-carbon compound formed by the oxidation of pyruvate, and not pyruvate itself, condenses with the oxalacetate. It seemed possible that acetyl phosphate might be the reactive 2-carbon compound, since it can be formed in pyruvate oxidation (Lipmann (22)) and might arise from acetoacetate in a reaction coupled with α-ketoglutarate oxidation. Ochoa (23) has shown that this oxidation can produce phosphate esterification. Acetyl phosphate was tested, but it did not produce any citrate formation. Addition of phosphate or adenosine triphosphate produced no increase in citrate formation; yet the intervention of phosphate in the reaction has not been entirely ruled out, for the amount of phosphate in the system was 3 to 4 $\times$ 10^{-3} M, sufficient for α-ketoglutarate oxidation to be 75 per cent of its maximum (23).

Although the trials with acetyl phosphate were negative, there is still a strong suggestion that α-ketoglutarate oxidation may be coupled with changes in the acetoacetate which prepare it for condensation with oxalacetate. In aerobic experiments without oxalacetate, α-ketoglutarate was oxidized and acetoacetate disappeared, but it did not appear either as β-hydroxybutyrate or as citrate. Under such conditions an unstable intermediate 2-carbon compound might be hydrolyzed to acetate.

Acetate is rapidly metabolized by kidney tissue and it has been suggested that it enters the tricarboxylic acid cycle (21). Rittenberg and Bloch (24) have recently provided additional evidence in favor of this idea. Acetate might enter the cycle directly or it might be synthesized to acetoacetate before conversion to citrate. Medes *et al.* (25) have recently demonstrated the synthesis of acetoacetate from acetate in kidney tissue. Acetate did not increase citrate formation in our preparations, but this could have been due to inactivity of the necessary enzymes.

SUMMARY

The formation of citric acid from acetoacetic acid and oxalacetic acid in kidney tissue has been studied. The necessary enzymes were found in the insoluble particles from homogenized kidney cortex. Citric acid was not oxidized by these preparations.

When oxalacetic acid and acetoacetic acid were added to the washed tissue particles, very little acetoacetic acid was removed and very little extra citric acid was formed unless α-ketoglutaric acid, glutamic acid, or glutathione was also added. The activity of glutamic acid is believed due to conversion to α-ketoglutaric acid. The activity of glutathione is believed due to hydrolysis to yield glutamic acid.

The evidence indicates that α-ketoglutaric acid was oxidized in both the aerobic and in the anaerobic experiments. In the first case oxygen was the ultimate oxidant; in the second case excess oxalacetic acid acted as the oxidant.

Since the conversion of acetoacetic acid to citric acid occurred only in experiments in which α-ketoglutaric acid (or glutamic acid) was being oxidized, this conversion must be linked to α-ketoglutaric acid oxidation in the preparations used.

Balance experiments with determinations of citric acid, acetoacetic acid, β-hydroxybutyric acid, oxygen consumption aerobically, and acid formation anaerobically were carried out. The results were essentially the same aerobically and anaerobically. About 2 molecules of extra citric acid were formed for each molecule of acetoacetic acid which disappeared. Reduction to β-hydroxybutyric acid did not account for the disappearance of acetoacetic acid. Approximately one extra acidic group was formed for each molecule of citric acid formed from pyruvic acid or from acetoacetic acid.

In aerobic experiments without oxalacetic acid there is still a removal of ketone bodies which is dependent on α-ketoglutaric acid oxidation, but very little citric acid is formed. It is suggested that the primary reaction may be the conversion of acetoacetic acid to an intermediate compound which condenses with oxalacetic acid, if present, to form the precursor of citric acid.

Modified methods are described for the estimation of citric acid and for acetoacetic acid in the presence of oxalacetic acid.

The authors are greatly indebted to Professor Carl F. Cori and Dr. S. P. Colowick for many valuable suggestions and criticisms.

BIBLIOGRAPHY

1. Breusch, F. L., *Science*, **97**, 490 (1943).
2. Wieland, H., and Rosenthal, C., *Ann. Chem*, **554**, 241 (1943).

3. Martius, C., *Z. physiol. Chem.*, **279**, 96 (1943).
4. Weil-Malherbe, H., *Nature*, **153**, 435 (1944).
5. Krebs, H. A., and Eggleston, L. V., *Nature*, **154**, 209 (1944).
6. Buchanan, J. M., Sakami, W., Gurin, S., and Wilson, D. W., *J. Biol. Chem.*, **157**, 747 (1945).
7. Krampitz, L. O., and Werkman, C. H., *Biochem. J.*, **35**, 595 (1941).
8. Ljunggren, G., *Biochem. Z.*, **145**, 422 (1924).
9. Lipmann, F., and Tuttle, L. C., *J. Biol. Chem.*, **153**, 571 (1944).
10. Robertson, W. B., *Science*, **96**, 93 (1942).
11. Edson, N. L., *Biochem. J.*, **29**, 2082 (1935).
12. Weichselbaum, T. E., and Somogyi, M., *J. Biol. Chem.*, **140**, 5 (1941).
13. Krebs, H. A., *Biochem. J.*, **36**, 303 (1942).
14. Pucher, G. W., Sherman, C. C., and Vickery, H. B., *J. Biol. Chem.*, **113**, 235 (1936).
15. Goldberg, A. S., and Bernheim, A. R., *J. Biol. Chem.*, **156**, 33 (1944).
16. Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, **114**, 495 (1936).
17. Woodward, G. E., and Reinhart, F. E., *J. Biol. Chem.*, **145**, 471 (1942).
18. Krebs, H. A., and Eggleston, L. V., *Biochem. J.*, **37**, 334 (1943).
19. Krebs, H. A., Eggleston, L. V., Kleinzeller, A., and Smyth, D. H., *Biochem. J.*, **34**, 1234 (1940).
20. Wood, H. G., Werkman, C. H., Hemingway, A., and Nier, A. O., *J. Biol. Chem.*, **139**, 483 (1941).
21. Krebs, H. A., in Nord, F. F., and Werkman, C. H., Advances in enzymology and related subjects, New York, **3**, 191 (1943).
22. Lipmann, F., *Federation Proc.*, **1**, pt. 2, 122 (1942).
23. Ochoa, S., *J. Biol. Chem.*, **155**, 87 (1944).
24. Rittenberg, D., and Bloch, K., *J. Biol. Chem.*, **157**, 749 (1945).
25. Medes, G., Weinhouse, S., and Floyd, N. F., *J. Biol. Chem.*, **157**, 751 (1945).

THE USE OF ACETOBACTER SUBOXYDANS FOR ASSAY OF THE LACTONE MOIETY OF PANTOTHENIC ACID*

By HERBERT P. SARETT† and VERNON H. CHELDELIN

(*From the Department of Chemistry, Oregon State College, Corvallis*)

(Received for publication, March 13, 1945)

Acetobacter suboxydans (1) and hemolytic streptococcus strain H69D (2) are the only known microorganisms for which the lactone[1] moiety of pantothenic acid, *l*-α-hydroxy-β,β-dimethyl-γ-butyrolactone, can replace pantothenic acid as an essential growth factor. Complete studies of the nutrition of *Acetobacter suboxydans* (1, 3–6) determined all of its essential growth requirements and showed that it could be used for the assay of *p*-aminobenzoic acid (4, 5) and nicotinic acid (6).

Although the lactone moiety can replace pantothenic acid for growth of the above organisms, β-alanine has no activity. Most animals and bacteria require the intact pantothenic acid molecule (7–10). Lactic acid bacteria cannot utilize lactone or β-alanine or a combination of both (9, 10). Several yeasts (11, 12) and the diphtheria bacillus (13) can utilize the β-alanine fragment but not the lactone.

In the present communication a method is proposed for the assay of lactone by the use of *Acetobacter suboxydans*. The sensitivity of the organism to lactone, as reported by Underkofler *et al.* (1), has been increased by changes in the medium and by the finding that the organism can utilize the alkali-hydrolyzed lactone, α,γ-dihydroxy-β,β-dimethylbutyric acid, 4 to 5 times as effectively as it does the intact lactone. Turbidimetric measurements after 60 to 70 hours permit measurement of 0.1 to 0.5 γ of the dihydroxy acid[1] per flask (10 ml.). For assay purposes all of the pantothenic acid and lactone present is hydrolyzed by alkali to the dihydroxy acid before measurement. Assay is also made for the pantothenic acid present in the original material. This method does not allow accurate measurement of small amounts of lactone in the presence of

* A preliminary report was presented before the Division of Biological Chemistry at the 108th meeting of the American Chemical Society, New York.

Published with the approval of the Monographs Publication Committee, Oregon State College. Research paper No. 90, School of Science, Department of Chemistry.

This work was supported by the Nutrition Foundation, Inc.

† Present address, Medical Research Laboratory, Edgewood Arsenal, Maryland.

[1] In the present communication, the lactone moiety of pantothenic acid refers to *levo*-α-hydroxy-β,-β-dimethyl-γ-butyrolactone. Alkali-hydrolyzed lactone or the dihydroxy acid refers to α,γ-dihydroxy-β,β-dimethylbutyric acid. We wish to thank Dr. J. C. Keresztesy of Merck and Company, Inc., for a gift of the lactone used in these studies.

excess pantothenic acid, but is useful in searching for lactone which is not bound as pantothenic acid, and in investigation of pantothenic acid metabolism (14).

Method

Acetobacter suboxydans[2] is maintained on slants of medium containing 0.5 per cent Difco yeast extract, 5 per cent glycerol, and 2 per cent agar. The slants are incubated at 30° for about 36 hours and are then kept in the refrigerator. Stock cultures are transferred every month and slants for daily use are prepared weekly. The test inoculum is obtained from a 30 to 40 hour culture grown at 30° in a 50 ml. Erlenmeyer flask in 10 ml. of the diluted basal medium (Table I), to which 0.1 γ of the dihydroxy acid and 5 mg. of liver concentrate have been added.

Basal Medium—The medium proposed by Underkofler *et al.* (1) and used for assay of *p*-aminobenzoic acid (4, 5) and nicotinic acid (6) does not respond to small amounts of the dihydroxy acid or pantothenic acid and requires 1 to 5 γ per flask to obtain a suitable growth curve. A comparison of this medium with the suggested basal medium is presented in Table I, and shows the changes which have been made to increase the growth response of the organism. The new medium permits the measurement of 0.1 to 0.5 γ of the dihydroxy acid per flask, gives approximately the same growth with the dihydroxy acid as with equimolecular amounts of pantothenic acid, and promotes as good growth with excess dihydroxy acid or pantothenic acid as is obtained in the presence of yeast or liver extracts.

Glucose (or its end-products after being autoclaved) has a marked stimulatory action as a carbohydrate source in addition to the glycerol present. Increasing the casein content of the medium to 10 gm. per liter also results in more rapid and heavier growth. Purines and pyrimidines, which were shown to be stimulatory for the *p*-aminobenzoic acid assay (5), are likewise necessary for maximum sensitivity to the dihydroxy acid. β-Alanine was added in amounts ranging from 1 γ to 2 mg. per flask, and was found to increase the growth obtained with small amounts of lactone or the dihydroxy acid. The stimulation obtained with 2 mg. per flask was about the same as was found with 10 γ per flask. The smaller amount was retained in the medium for its growth promotion and to offset the effect of any β-alanine which may be present as such or from hydrolysis of pantothenic acid in assay samples.

In the presence of the above supplements, the greatest response to the

[2] This organism is listed as No. 621 in the American Type Culture Collection. Dr. L. A. Underkofler of the University of Wisconsin kindly supplied us with a slant of the organism.

added dihydroxy acid was obtained by the inclusion in the medium of charcoal-treated liver extract and charcoal-treated peptone. These pantothenic acid-free extracts are prepared together as follows:

10 gm. of liver concentrate[3] and 25 gm. of Difco peptone are dissolved in water, diluted to a volume of 500 ml., steamed for 10 minutes, and cooled. The pH is adjusted to 1.5 and the solution shaken for 20 minutes with

TABLE I

Basal Media for Acetobacter Suboxydans

	Assay for *p*-aminobenzoic acid (*cf.* (4, 5))	Present medium
	gm.	*gm.*
Glycerol	100	100
Glucose		5
Casein hydrolysate	6	10
Norit A-treated peptone*		5
" " liver concentrate*		2
	mg.	*mg.*
Tryptophane	200	200
Cystine	150	150
Adenine	10	10
Guanine	10	10
Xanthine	10	
Uracil		10
β-Alanine		2
	ml.	*ml.*
Salt Solution A†	10	10
B†	10	10
	γ	γ
Calcium pantothenate	200	
Nicotinic acid	200	200
p-Aminobenzoic acid		200
Distilled water to 1 liter, pH 6.0		

* See the text for preparation.

† Prepared according to Snell and Strong (15).

35 gm. of norit A and filtered. The pH is readjusted to 1.5 and the charcoal treatment repeated. The second filtrate is adjusted to pH 6.0, steamed, cooled, and filtered. The final solution, containing 50 mg. of peptone and 20 mg. of liver concentrate per ml., is kept in the refrigerator under toluene.

[3] We are indebted to Dr. T. H. Jukes of the Lederle Laboratories, Inc., for a gift of liver concentrate and to Stanley W. Hier of The Wilson Laboratories for a sample of Wilson's 1:20 liver powder. Either of these preparations may be used.

No further improvements in the medium could be made by changing the pH or by adding other known B vitamins, *dl*-alanine, asparagine, succinic acid, sodium bicarbonate, ammonium sulfate, sodium acetate, or norit-treated yeast or beef extracts.

The presence of 2 mg. of *dl*-alanine per flask inhibits utilization of the lactone or the dihydroxy acid but not of pantothenic acid. The same amount of asparagine curtails growth with all three of these substances. 2 mg. of taurine, the sulfur analogue of β-alanine, severely inhibit the growth response to all three forms of the growth factor. The inhibition of the split-products is greater than that of the intact pantothenic acid molecule, and is completely overcome by excess β-alanine. Experiments with pantoyltaurine showed sporadic inhibition or stimulation with 0.5 to 2 mg. per flask. The stimulatory effect seems to indicate that some of the pantoyltaurine was hydrolyzed, either in our sample or by the bacteria.

Assay Procedure—All tests are carried out in 50 ml. Erlenmeyer flasks. The sample (at pH 6.0) in each flask is diluted (if necessary) to 5 ml. with distilled water and 5 ml. of the basal medium (pH 6.0) shown in Table I are added. A standard curve which is run each time contains 0.0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, and 0.8 γ of the alkali-hydrolyzed lactone or its equivalent of alkali-hydrolyzed pantothenic acid. A few points are run in duplicate. The lactone standard is hydrolyzed by steaming or autoclaving 50 to 100 γ of lactone in 5 ml. of 1 N NaOH for 10 to 40 minutes, neutralizing to pH 6.0, and diluting to an appropriate volume. The same results are obtained after steaming or autoclaving of the lactone. When pantothenic acid is used, it must be autoclaved about 30 to 40 minutes. Samples to be assayed are run in duplicate at three levels, 1 to 3 ml. containing about 0.1 γ of lactone per ml.

All values in the present communication are in terms of the weight of the lactone. The molecular weight of the lactone is 130 or 54.6 per cent of the molecular weight (238) of the calcium pantothenate standard.

The flasks are plugged and autoclaved for 15 minutes. After being cooled, each flask is inoculated with 1 drop of the inoculum, prepared by transferring the 30 to 40 hour culture previously described to a sterile test-tube, centrifuging, and resuspending the organisms in about 15 ml. of sterile saline. The flasks are incubated in a 30° water bath for 60 to 70 hours. Convenient wooden holders have been devised, each of which holds a row of flasks in the bath. Growth is measured turbidimetrically after shaking each culture thoroughly and transferring the suspension to a test-tube. The turbidities are read with a 540 mμ filter in any reliable photoelectric colorimeter, with an uninoculated culture as a blank. The turbidimetric readings are given in terms of optical density which is equal to log 100 minus log per cent transmission.

A typical standard curve for the dihydroxy acid is shown in Fig. 1. The growth curve obtained with equimolecular amounts of pantothenic acid is approximately the same. The untreated lactone, or lactone formed by acid treatment of the dihydroxy acid, has about 20 to 25 per cent of the activity of the dihydroxy acid. In order to lactonize the dihydroxy acid, it is necessary to heat the solution at 100° for 10 minutes at a pH of 2 or less.

Preparation of Sample for Assay—Several attempts were made to separate the lactone from pantothenic acid quantitatively in order to avoid the difficulties inherent in determining total lactone and pantothenic acid and

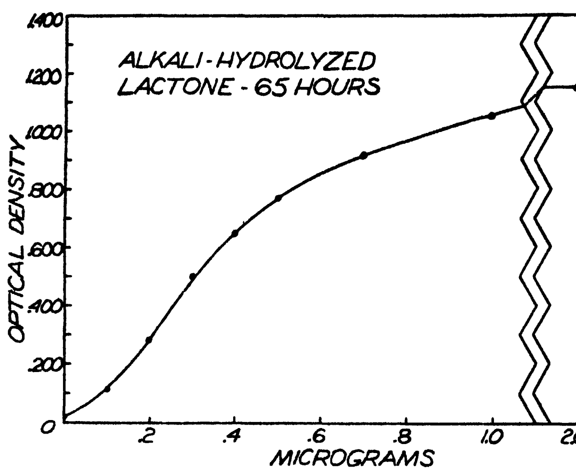


FIG. 1. Growth response of *Acetobacter suboxydans* to the alkali-hydrolyzed lactone moiety of pantothenic acid. The weights shown are in terms of the lactone. Optical density is log 100 minus log per cent transmission (2 − log G).

obtaining values by difference. All of the charcoals tried removed lactone as well as pantothenic acid from solution. Other experiments with adsorbents and ion exchange resins were unsuccessful. Ether extraction of lactone from an aqueous solution at pH 7 to 7.5 (16) was not quantitative for the low concentrations of lactone encountered. Only 30 to 40 per cent could be recovered in this manner. Since it was considered possible that the dihydroxy acid as well as the lactone might occur naturally, and that the acid would not be extracted by the ether, these experiments were abandoned. The finding that the dihydroxy acid was 4 to 5 times as active as the lactone led to the use of alkali-hydrolyzed samples, with appropriate corrections for the pantothenic acid in the samples.

2 gm. samples are autoclaved in 10 ml. of 1 N NaOH for 30 to 40 minutes, cooled, neutralized to pH 6.0, diluted to proper volume, and filtered if

necessary. For analysis an aliquot is diluted so that 1 ml. contains about 0.1 γ of lactone. Extracts of samples containing large amounts of pantothenic acid or lactone are usually clear and colorless. For slightly turbid or colored solutions, an uninoculated flask containing the largest amount of the diluted sample is set up and used as a blank correction for the "top" flask. Simple division of this blank value gives correction figures for the other flasks of the series. Pantothenic acid assays are carried out on the original material by the method of Hoag, Sarett, and Cheldelin (17).

TABLE II

Total Lactone Analyses and Recoveries

Material	Sample	Lactone found	Lactone per gm. or ml.	Recovery
	mg.	γ	γ	*per cent*
Yeast-liver extract	0.67	0.19	285	
	1.33	0.34	255	
	2.0	0.54	270	
	0.67 + 0.2 γ lactone	0.38		95
	1.33 + 0.2 " "	0.54		100
Rice bran extract	1	0.12	120	
	2	0.25	125	
	3	0.37	123	
	4	0.52	130	
	1 + 0.2 γ lactone	0.32		100
	2 + 0.2 " "	0.43		90
Urine C2			8.0	
" " + 25 γ pantothenic acid* per ml.			21.8	102
" R2			13.4	
" " + 25 γ pantothenic acid* per ml.			27.4	103
" S2			8.0	
" " + 25 γ pantothenic acid* per ml.			21.3	98

* 25 γ pantothenic acid contain 13.6 γ of lactone (54.6 per cent).

Table II shows the lactone value and recoveries of added lactone or pantothenic acid for some samples. There is good agreement at the different levels assayed and good recoveries of lactone, added either as lactone or pantothenic acid. The pantothenic acid recovered in the three urine samples was added to the urines before alkaline hydrolysis and carried through the entire assay.

Results

The total lactone contents as measured by *Acetobacter suboxydans* and that calculated from pantothenic acid assays are shown in Table III for

nine samples. Four of the samples had been analyzed for pantothenic acid by both microbiological and chick methods. For most of the samples the total lactone was greater than that which could be accounted for by microbiological pantothenic acid assay. Since most of them were concentrates, it was natural to expect some pantothenic acid breakdown to lactone or the dihydroxy acid. No extra lactone was found in the ground wheat or the rice bran extracts.

The pantothenic acid values obtained for certain foods by chick assay have been higher than those reported by microbiological assay (17–19). The yeast concentrates shown in Table III do not contain enough total lactone to account for the high chick values, and it appears that the chick values are not due solely to the pantothenic acid content of the concentrates.

Table III

Total Lactone and Lactone Calculated from Pantothenic Acid Assays

Sample	Total lactone	Pantothenic acid lactone,* microbiological	Pantothenic acid lactone,* chick assay†
	γ per gm.	*γ per gm.*	*γ per gm.*
Yeast-liver Concentrate 1	140	96	267
" " 2	135	108	333
Fortified yeast-liver concentrate	275	255	435
Rice bran extract 1	125		120
" " " 2	150	149	153
Ground wheat	3.7	3.6	
Skim milk powder	24.5	12.5	
Yeast concentrate	85	46	
Liver powder (Wilson's 1:20)	307	169	

* Calculated from the pantothenic acid found (54.6 per cent).

† We are indebted to Dr. A. L. Caldwell of Eli Lilly and Company for the chick assays.

Neal and Strong (18) and Willerton and Cromwell (19) attributed the difference between microbiological and chick assays to poor digestion of the sample before the microbiological assay. By incubating 0.5 gm. of the samples with 1 gm. of clarase in 10 ml. of buffer for 48 hours, Willerton and Cromwell (19) obtained microbiological values which approached the chick values. Neal and Strong (18) found that, after strong alkaline hydrolysis of yeast, liver, and other substances, some pantothenic acid action remained. For liver the residual activity was 4 times as great when measured by chick assay as when measured microbiologically. Since differences between chick and microbiological assays are only found in certain types of foods, it is possible that the chick diet may be incomplete. The discrepancies reported here between total lactone figures and

chick assays for pantothenic acid suggest that factors other than pantothenic acid are influencing the chick values.

SUMMARY

A microbiological assay procedure for the lactone portion of the pantothenic acid molecule is presented, based upon the growth response of *Acetobacter suboxydans*. Alkaline cleavage of the lactone to α,γ-dihydroxy-β,β-dimethylbutyric acid increases its growth-promoting effect several fold and makes it as effective as equimolecular amounts of pantothenic acid. Supplements have been added to the medium which allow measurement of 0.1 to 0.5 γ of lactone per flask.

β-Alanine cannot replace pantothenic acid or the dihydroxy acid for growth, but increases the response to the dihydroxy acid and is included in the medium. Taurine inhibits growth response to both the dihydroxy acid and pantothenic acid. This inhibition may be overcome by excess β-alanine.

For assay purposes alkaline hydrolysis is used to convert lactone and pantothenic acid to the dihydroxy acid. Good agreement of total lactone values of samples assayed at different levels and complete recovery of added lactone or pantothenic acid have been obtained for several foods, concentrates, and urines.

Yeast concentrates were found to contain more lactone than could be accounted for by microbiological assays for pantothenic acid, but not enough lactone to account for the high values obtained by chick assay.

BIBLIOGRAPHY

1. Underkofler, L. A., Bantz, A. C., and Peterson, W. H., *J. Bact.*, **45**, 183 (1943).
2. Woolley, D. W., *J. Biol. Chem.*, **130**, 417 (1939).
3. Lampen, J. O., Underkofler, L. A., and Peterson, W. H., *J. Biol. Chem.*, **146**, 277 (1942).
4. Landy, M., and Dicken, D. M., *J. Biol. Chem.*, **146**, 109 (1942).
5. Landy, M., and Streightoff, F., *Proc. Soc. Exp. Biol. and Med.*, **52**, 127 (1943).
6. Karabinos, J. V., and Dicken, D. M., *Arch. Biochem.*, **4**, 211 (1944).
7. Oleson, J. J., Woolley, D. W., and Elvehjem, C. A., *Proc. Soc. Exp. Biol. and Med.*, **42**, 151 (1939).
8. Woolley, D. W., Waisman, H. A., and Elvehjem, C. A., *J. Am. Chem. Soc.*, **61**, 977 (1939).
9. Snell, E. E., Strong, F. M., and Peterson, W. H., *J. Bact.*, **38**, 293 (1939).
10. Cheldelin, V. H., Hoag, E. H., and Sarett, H. P., *J. Bact.*, **49**, 41 (1945).
11. Williams, R. J., and Rohrmann, E., *J. Am. Chem. Soc.*, **58**, 695 (1936).
12. Sarett, H. P., and Cheldelin, V. H., *J. Bact.*, **49**, 31 (1945).
13. Mueller, J. H., and Cohen, S., *J. Bact.*, **34**, 381 (1937).
14. Sarett, H. P., *J. Biol. Chem.*, **159**, 321 (1945).
15. Snell, E. E., and Strong, F. M., *Ind. and Eng. Chem., Anal. Ed.*, **11**, 346 (1939).

16. Woolley, D. W., Waisman, H. A., and Elvehjem, C. A., *J. Biol. Chem.*, **129**, 673 (1939).
17. Hoag, E. H., Sarett, H. P., and Cheldelin, V. H., *Ind. Eng. and Chem., Anal. Ed.*, **17**, 60 (1945).
18. Neal, A. L., and Strong, F. M., *J. Am. Chem. Soc.*, **65**, 1659 (1943).
19. Willerton, E., and Cromwell, H. W., *Ind. and Eng. Chem., Anal. Ed.*, **14**, 603 (1942).

THE METABOLISM OF PANTOTHENIC ACID AND ITS LACTONE MOIETY IN MAN*

By HERBERT P. SARETT†

(*From the Department of Chemistry, Oregon State College, Corvallis*)

(Received for publication, March 13, 1945)

The daily urinary excretion of pantothenic acid has been found to average 3.1 to 3.8 mg. for a few groups of normal subjects (1–4). Some data have been published which show that only a small part of test doses of pantothenic acid is excreted in the urine (1, 5) but no information is available on the fate of the remainder (6).

Since certain bacteria and many strains of yeast can utilize the lactone[1] moiety (7–9) or β-alanine (10–12) to replace pantothenic acid for growth, the present study was undertaken to determine whether the lactone moiety is a breakdown product in the metabolism of pantothenic acid by humans. Pantothenic acid and total lactone (after alkaline hydrolysis) were measured in normal urines, and after oral and intravenous doses of pantothenic acid, lactone, and alkali-hydrolyzed lactone.[1] The results show that lactone is not a normal constituent of urine, does not account for the pantothenic acid which disappears after test doses, and is not metabolized by humans.

EXPERIMENTAL

The experiments were conducted on three male subjects, aged 23 to 29, each of whom continued on his regular diet throughout the course of the experiments (10 weeks) with no vitamin B supplements. Test doses were given at about weekly intervals and 24 hour urines were collected with 10 ml. of glacial acetic acid and preserved in the refrigerator. Intravenous injections were made with freshly sterilized solutions of pantothenic acid or lactone in 5 ml. of saline. Alkali-hydrolyzed lactone was prepared by autoclaving 160 mg. of lactone in 5 ml. of 0.5 N NaOH (9). Neutralization with HCl and dilution to 16 ml. provided the proper saline concentration for administration.

* Published with the approval of the Monographs Publication Committee, Oregon State College. Research paper No. 91, School of Science, Department of Chemistry. This work was supported by the Nutrition Foundation, Inc.

† Present address, Medical Research Laboratory, Edgewood Arsenal, Maryland.

[1] In the present communication, the lactone moiety of pantothenic acid refers to *levo*-α-hydroxy-β,β-dimethyl-γ-butyrolactone. Alkali-hydrolyzed lactone or the dihydroxy acid refers to α,γ-dihydroxy-β,β-dimethylbutyric acid. We wish to thank Dr. J. C. Keresztesy of Merck and Company, Inc., for a gift of the lactone used in these studies.

The pantothenic acid of the test urines was measured by the use of *Lactobacillus arabinosus* (13), and total lactone (after alkaline hydrolysis

TABLE I

Urinary Excretion of Pantothenic Acid and Total Lactone by Three Subjects on Normal Diets and after Test Doses

	Subject and sample No.	Panto-thenic acid	Panto-thenic acid lactone*	Total lactone	Total lactone minus panto-thenic acid lactone*
		mg.	*mg.*	*mg.*	*mg.*
Control	C-1	1.3	0.7	0.8	0.1
	R-1	3.2	1.7	1.7	0.0
	S-1	4.3	2.3	2.6	0.3
	C-5	5.3	2.9	2.8	−0.1
	R-5	2.7	1.5	1.8	0.3
	S-5	4.0	2.2	2.1	−0.1
After 100 mg. pantothenic acid, orally	C-2	12.1	6.6	12.7	6.1
	R-2	21.0	11.5	13.8	2.3
	S-2	17.0	9.3	13.3	4.0
	C-7	22.0	12.0	12.6	0.6
	R-7	26.4	14.4	15.6	1.2
	S-7	18.9	10.3	12.8	2.5
	C-9	18.9	10.3	11.8	1.5
	R-9	11.7	6.4	9.4	3.0
	S-9	17.9	9.8	11.9	2.1
" 100 " " " intravenously	C-6	37.6	19.9	19.9	0.0
	R-6	43.9	23.9	23.8	−0.1
	S-6	26.4	14.4	14.7	0.3
After 50 mg. lactone, intravenously	C-3	4.6			
	R-3	3.6	2.0	52.7	50.7
	S-3	4.4	2.4	53.7	51.3
" 50 " " orally	C-8	7.2	3.9	51.0	47.1
	R-8	6.2	3.4	52.7	49.3
	S-8	5.3	2.9	51.2	48.3
	C-10	8.4	4.6	57.0	52.4
	R-10	5.8	3.2	55.2	52.0
	S-10	4.5	2.5	54.0	51.5
After 50 mg. alkali-hydrolyzed lactone, intravenously	C-4	5.7	3.1	51.9	48.8
	R-4	3.0	1.6	53.0	51.4
	S-4	2.4	1.3	54.2	52.9
After 50 mg. alkali-hydrolyzed lactone, orally	C-11	6.3	3.4	41.8	38.4
	R-11	6.6	3.6	52.0	48.4
	S-11	5.0	2.7	41.3	38.6

* Calculated from the pantothenic acid found (54.6 per cent).

of the urines) assayed with *Acetobacter suboxydans* (9). All lactone and dihydroxy acid values are reported on the basis of the lactone, whose molec-

ular weight is 130, which is 54.6 per cent of the molecular weight of the calcium pantothenate used as the standard for pantothenic acid (9).

Results

The excretion of pantothenic acid and total lactone in normal urines and after various test doses is shown in Table I. The pantothenic acid content (*Lactobacillus arabinosus*) of the normal urines ranged from 1.3 to 5.3 mg. (average, 3.5 mg.) and accounted for approximately all of the lactone content, as measured by *Acetobacter suboxydans*. The average pantothenic acid excretions of these control days were used in the calculation of additional pantothenic acid excretion after test doses.

After oral administration of 100 mg. of pantothenic acid, the three subjects excreted 8.8 to 23.4 mg. of extra pantothenic acid, averaging 15 mg. However, the pantothenic acid content of these urines did not account for the total lactone excretion. The urines contained from 0.6 to 6.1 mg. of lactone in addition to that calculated from the pantothenic acid content. The average for the nine urines was 2.6 mg. of extra lactone. Intravenous administration of 100 mg. of pantothenic acid resulted in greater pantothenic acid excretion (22.3 to 40.9 mg., average 35 mg.) but in contrast to the oral test dose, no extra lactone was detectable in the urine.

50 mg. doses of lactone taken orally or intravenously could be completely recovered in the urine. After the oral dose of lactone, the pantothenic acid content of the urine increased to an average of 6.2 mg. per day.

Since *Acetobacter suboxydans* was found to utilize the alkali-hydrolyzed lactone, α,γ-dihydroxy-β,β-dimethylbutyric acid, 4 to 5 times as effectively as the lactone (9), the dihydroxy acid form was tested on the three subjects. Intravenous administration of the dihydroxy acid resulted in its total excretion in the urine, but after oral doses the excretion by two of the subjects was only about 80 per cent of the dose. The oral administration of the dihydroxy acid, like that of the lactone, increased the pantothenic acid excretion to an average of 6.0 mg.

DISCUSSION

Although some bacteria can synthesize pantothenic acid from its lactone moiety, the evidence presented here indicates that the lactone portion of the pantothenic acid molecule is not an intermediate in the breakdown of pantothenic acid by man. Since the three subjects did not metabolize the lactone or the dihydroxy acid, and since none of these compounds appeared in the urine after an intravenous dose of pantothenic acid, the breakdown of pantothenic acid in humans must proceed by one or more other pathways.

The excretion of a little extra lactone or dihydroxy acid after oral doses of pantothenic acid appears to be due to the action of intestinal bacteria.

Bacteria may also have some effect upon the lower excretion of pantothenic acid after oral doses compared to intravenous doses of pantothenic acid. However, the high blood levels of pantothenic acid after intravenous injection may account for most of the difference (1).

Since the excretion of lactone is the same after oral or intravenous administration of lactone, it appears that the intestinal bacteria do not attack the lactone ring. However, the lower excretion of oral doses of the dihydroxy acid seems to indicate some bacterial action on this compound. Both of these compounds are completely recovered after intravenous administration.

The increases in pantothenic acid excretion after oral doses of the lactone or of dihydroxy acid are not readily explainable, but may possibly result from a stimulatory action of this molecular configuration upon the intestinal bacteria. This effect is not observed with intravenous doses of these compounds.

SUMMARY

The pantothenic acid and total lactone content has been measured in urines of three male subjects on normal diets, and after oral and intravenous administration of pantothenic acid, and its lactone or dihydroxy acid moieties.

The normal pantothenic acid excretion averaged 3.5 mg. per day and accounted for the total lactone present, as measured after alkaline hydrolysis of the urine.

After 100 mg. oral doses of pantothenic acid an average of 15 mg. of additional pantothenic acid and 2.6 mg. of extra lactone were found in the urines. Intravenous administration of the same amount of pantothenic acid resulted in the excretion of 35 mg. of additional pantothenic acid. This accounted for all of the lactone present.

Oral or intravenous doses of 50 mg. of lactone and intravenous doses of 50 mg. of the dihydroxy acid were completely recovered in the urine. After oral doses of the dihydroxy acid, only 80 per cent was found in two of the urines. After oral doses of the lactone or of the dihydroxy acid, the daily pantothenic acid excretion increased to about 6 mg.

It is concluded that the lactone or the dihydroxy acid moieties are not normal intermediates in the metabolism of pantothenic acid in man.

The author wishes to thank Dr. V. H. Cheldelin for his advice and suggestions.

BIBLIOGRAPHY

1. Pearson, P. B., *Am. J. Physiol.*, **135**, 69 (1941).
2. Pelczar, M. J., and Porter, J. R., *Proc. Soc. Exp. Biol. and Med.*, **47**, 3 (1941).
3. Krahnke, H., and Gordon, E. S., *J. Am. Med. Assn.*, **116**, 2431 (1941).

4. Wright, L. D., and Wright, E. Q., *Proc. Soc. Exp. Biol. and Med.*, **49**, 80 (1944).
5. McLean, R. A., Smith, D. T., and Smith, S. G., Abstracts, American Chemical Society, 108th meeting, New York, 11B (1944).
6. Lustig, B., Goldfarb, A. R., and Gerstl, B., *Arch. Biochem.*, **5**, 59 (1944).
7. Underkofler, L. A., Bantz, A. C., and Peterson, W. H., *J. Bact.*, **45**, 183 (1943).
8. Woolley, D. W., *J. Biol. Chem.*, **130**, 417 (1939).
9. Sarett, H. P., and Cheldelin, V. H., *J. Biol. Chem.*, **159**, 311 (1945).
10. Williams, R. J., and Rohrmann, E., *J. Am. Chem. Soc.*, **58**, 695 (1936).
11. Sarett, H. P., and Cheldelin, V. H., *J. Bact.*, **49**, 31 (1945).
12. Mueller, J. H., and Cohen, S., *J. Bact.*, **34**, 381 (1937).
13. Hoag, E. H., Sarett, H. P., and Cheldelin, V. H., *Ind. and Eng. Chem., Anal. Ed.*, **17**, 60 (1945).

RELATION OF SCURVY TO THE ADRENALIN CONTENT OF THE ADRENAL GLANDS OF GUINEA PIGS

BY SACHCHIDANANDA BANERJEE*

(From the Department of Mitra Research in Diabetes, School of Tropical Medicine, Calcutta, India)

(Received for publication, March 29, 1945)

It has been reported from this laboratory (1) that a deficiency of vitamin C in guinea pigs leads to changes in carbohydrate metabolism, as evidenced by glycosuria, a diabetic type of the glucose tolerance curve, and depletion of the glycogen content of the liver. We have also found that there is a fall in the insulin content of the pancreas (2), an increase in the size and also the number of islets, and degranulations of the β-cells of the pancreas (3) of scorbutic guinea pigs.

It is well known that adrenalin and insulin produce opposite glycemic effects (Cori and coworkers (4, 5)). It was therefore of particular interest to examine the effect of scurvy on the adrenalin content of the adrenal glands of guinea pigs.

McCarrison (6) reported that the adrenalin content of the adrenals of scorbutic guinea pigs is decreased. Ohata (7) also noted a definite reduction in the adrenalin content of the adrenal glands of scorbutic guinea pigs and presented evidence that this was not purely an effect of inanition. Deutsch and Schlapp (8) likewise reported a decrease in the adrenalin content of the adrenal glands of guinea pigs in scurvy. Mouriquand and Leulier (9), however, found no lowering of the adrenalin content of the adrenals of guinea pigs in avitaminosis C. Guha (10) also observed no change in the adrenalin content of the adrenals of scorbutic guinea pigs.

In view of the conflicting data cited above it was decided to reinvestigate the adrenalin content of the adrenal glands of scorbutic guinea pigs. A preliminary report of this work has been published elsewhere (11).

EXPERIMENTAL

Two groups of young guinea pigs of weights varying between 250 gm. and 400 gm. were selected. One of the groups was fed *ad libitum* a scorbutic diet consisting of crushed barley 64 parts, crushed gram[1] 20 parts, casein 12 parts, calcium carbonate 3 parts, and common salt 1 part for 22 to 25

* Present address, Department of Biochemistry, Cornell University Medical College, New York.

[1] *Cicer arietinum* (Leguminosae) is a pulse commonly cultivated as human food in India.

days until the animals developed scurvy. The second group of guinea pigs received the above diet for 15 days with the daily oral supplement of 5 mg. of ascorbic acid per animal. The animals of both the groups were fed 0.2 cc. of a concentrate of vitamins A and D (adexoline, Glaxo) twice a week during the experimental period.

TABLE I

Determination of Adrenalin in Normal and Scorbutic Guinea Pigs

	No. of animals	Average weight per animal	Weight of pooled adrenals	Weight of adrenal per 100 gm. body weight	Ascorbic acid per 1 gm. of adrenal	Adrenalin per 1 gm. of adrenal
		gm.	*mg*	*mg.*	*mg.*	γ
Scorbutic	4	310	1140	91	0.032	859
	4	374	1120	93	0.038	675
	6	292	1700	96	0.021	707
	7	340	2840	119	0.048	636
Mean				100	0.035	719.2
Normal	4	311	720	57	0.915	502
	4	310	860	69	0.732	230
	4	237	560	59	0.828	459
	4	323	760	59	1.184	263
	4	397	980	62	1.351	315
Mean.				61 2	1.002	353.8

TABLE II

Statistical Analysis

All values of *t* are highly significant.

	Weight of adrenal per 100 gm. body weight	Ascorbic acid per 1 gm. of adrenal	Adrenalin per 1 gm. of adrenal
	mg.	*mg.*	γ
Difference of means	38.8	0.967	365.4
Standard error of difference .	6.187	0.13078	74.66
t	6.27	7.39	4.89

The animals were fasted overnight. They were killed by a blow on the head and the neck veins were cut. The adrenals were carefully freed from the connective tissue and transferred to a weighed bottle containing normal saline and a few drops of 10 per cent trichloroacetic acid. The adrenal glands of several animals were pooled and extracted with trichloroacetic acid by the method of Barker and Marrian (12).

Of the different chemical methods for the estimation of adrenalin, Folin and Trimble's reagent (13) as used by Barker *et al.* (14) was found to be most satisfactory in our hands. It was shown by Guha (10) and Birch *et al.* (15) that Folin's tungstic acid reagent gives a blue color with ascorbic acid as well as with adrenalin. Rees (16) observed that the blue color obtained with Folin's reagent was proportional to the amount of ascorbic acid present. When the ascorbic acid in the adrenal gland extracts was first determined by titration with 2,6-dichlorophenol indophenol and the blue value corresponding to the amount of ascorbic acid present was subtracted from the total blue value of the sample, the true adrenalin content of the sample was obtained. This result was found by Rees to correspond to that determined biologically. Von Euler (17) and Schild (18) also obtained no significant difference in the adrenalin content of the adrenals of guinea pigs when it was determined by the biological and colorimetric methods. Rees' modified method for the colorimetric estimation of adrenalin was therefore used in the present investigation on the trichloroacetic acid extract of adrenal glands. The results are shown in Table I and the statistical analysis is given in Table II.

DISCUSSION

It will be noted from the data of Table I that the adrenal glands of the scorbutic guinea pigs were found to be much higher in adrenalin than were those of normal control animals. This is in contrast to the findings of previous investigators cited earlier. It is therefore of interest to compare the method used in this study with those used by others.

McCarrison (6) used Folin's reagent for the estimation of adrenalin and, since it is known (10, 15, 16) that Folin's reagent also gives a blue color with ascorbic acid, a relatively higher adrenalin value for the adrenals of normal guinea pigs might have resulted from the fact that the normal glands contain larger amounts of ascorbic acid. In scurvy the ascorbic acid content is greatly diminished (Table I), so that the diminution of the adrenalin value might have been apparent rather than real. Ohata (7), who also noted a reduction of the adrenalin content in scurvy, estimated adrenalin with iodic acid which likewise reacts with ascorbic acid. On the other hand Mouriquand and Leulier (9) who used mercuric chloride, which gives a red coloration with adrenalin and no coloration with ascorbic acid, obtained no decrease in the adrenalin content of the adrenal glands in scurvy. Guha (10) who determined adrenalin biologically also found no difference in the adrenalin content under the two conditions. These workers, however, used guinea pigs which were in the extreme stages of scurvy. On the other hand, our animals were killed when they were just falling in weight and were in the early stages of scurvy. Deutsch and

Schlapp (8) determined adrenalin content biologically and found a reduction in the adrenalin content of the adrenals in scurvy. These results which differ from that of Guha (10) are difficult to reconcile with our own. We do not know whether the different diets used by us had anything to do with it. The size of the adrenal glands has been found to be considerably increased in scurvy. This was also observed by Deutsch and Schlapp (8), McCarrison (6), Michaud (19), and Baldwin *et al.* (20). We have not used the paired feeding technique in our experiments and it might be argued that the results are due to inanition. However, in the experiments of Baldwin *et al.* (20) the paired feeding technique was employed and, although the adrenalin content of the adrenals was not determined, they observed the same increase in the weight of the gland as we have observed. It seems unlikely, therefore, that the increase in the adrenalin content in scurvy is the result of inanition. The increase in the adrenalin content of the adrenals in scurvy is of particular interest in relation to our earlier finding (2) that the insulin content of the pancreas is decreased. The action of vitamin C on the secretion of insulin appears to be specific in some degree, as the insulin content of the pancreas is not altered in vitamin B_1 deficiency, which also affects carbohydrate metabolism (21).

The lowered glucose tolerance and the lower glycogen content of the liver of the scorbutic guinea pigs as compared to normal animals may be partly due to an increased action of adrenalin in the absence of the opposing action of insulin. The lower glycogen content of the liver in scurvy might also be due in part to the decrease in adrenal cortical hormone in scorbutic guinea pigs observed by Giroud *et al.* (22, 23), since adrenal cortical hormone has been shown by Grattan and Jensen (24) to promote the deposition of glycogen in the liver.

SUMMARY

The effect of scurvy on the adrenalin content of the adrenal glands of guinea pigs was studied. A significant increase in the adrenalin content of the adrenals of scorbutic guinea pigs was noted, in contrast to the earlier findings of a diminution of the insulin content of the pancreas in scurvy.

Standard adrenalin was obtained by courtesy of Dr. B. Mukerji, director of the Biological Standardisation Laboratory, Government of India. Hoffmann-La Roche, Inc., made a free gift of 2,6-dichlorophenol indophenol.

BIBLIOGRAPHY

1. Banerjee, S., *Ann. Biochem. and Exp. Med.*, **3**, 157, 165 (1943).
2. Banerjee, S., *Nature*, **152**, 329 (1943); *Ann. Biochem. and Exp. Med.*, **4**, 33 (1944).
3. Banerjee, S., *Nature*, **153**, 344 (1944); *Ann. Biochem. and Exp. Med.*, **4**, 37 (1944).

4. Cori, C. F., and Cori, G. T., *J. Biol. Chem.*, **79**, 309 (1928).
5. Cori, G. T., Cori, C. F., and Buchwald, K. W., *J. Biol. Chem.*, **86**, 375 (1930).
6. McCarrison, R., *Brit. Med. J.*, **2**, 200 (1919).
7. Ohata, S., *J. Biochem.*, Japan, **12**, 419 (1930).
8. Deutsch, W., and Schlapp, W., *J. Physiol.*, **83**, 478 (1935).
9. Mouriquand, G., and Leulier, A., *Compt. rend. Soc. biol.*, **96**, 548 (1927).
10. Guha, B. C., *Sc. and Culture*, **1**, 111 (1935).
11. Banerjee, S., *Nature*, **153**, 526 (1944).
12. Barker, L. C., and Marrian, G. F., *Biochem. J.*, **21**, 1005 (1927).
13. Folin, O., and Trimble, H., *J. Biol. Chem.*, **60**, 473 (1924).
14. Barker, J. H., Estland, C. H., and Evers, N., *Biochem. J.*, **26**, 2129 (1932).
15. Birch, T. W., Harris, L. J., and Ray, S. N., *Biochem. J.*, **27**, 590 (1933).
16. Rees, H. G., *Quart. J. Pharm. and Pharmacol.*, **9**, 659 (1936).
17. von Euler, U. S., *J. Physiol.*, **78**, 462 (1933).
18. Schild, H., *J. Physiol.*, **79**, 455 (1933).
19. Michaud, A., *Bull. Soc. hyg. aliment.*, **18**, 44 (1930).
20. Baldwin, A. R., Longenecker, H. E., and King, C. G., *Arch. Biochem.*, **5**, 137 (1944).
21. Best, C. H., Haist, R. E., and Ridout, J. H., *J. Physiol.*, **97**, 137 (1939).
22. Giroud, A., Santa, N., and Martinet, M., *Compt. rend. Soc. biol.*, **134**, 23 (1940).
23. Giroud, A., Santa, N., Martinet, M., and Bellon, M. T., *Bull. Soc. chim. biol.*, **23**, 108 (1941).
24. Grattan, J. F., and Jensen, H., *J. Biol. Chem.*, **135**, 511 (1940).

THE FUNCTION OF PYRIDOXINE DERIVATIVES: ARGININE AND GLUTAMIC ACID DECARBOXYLASES

By W. W. UMBREIT and I. C. GUNSALUS

(*From the Laboratory of Bacteriology, College of Agriculture, Cornell University, Ithaca*)

(Received for publication, March 26, 1945)

Pyridoxal, the 4-aldehyde of pyridoxine described by Snell (1), has been shown to be converted into the coenzyme of tyrosine decarboxylase by living cells (2) and by enzyme preparations (3) of *Streptococcus faecalis*. With the latter a supply of adenosine triphosphate (ATP) is necessary (3). Gale and Epps (4, 5) have isolated a coenzyme for tyrosine (and lysine) decarboxylase and have reported that this substance, termed *codecarboxylase*, is free from phosphorus. Treatment of pyridoxal with chemical phosphorylating agents yields a preparation which activates the tyrosine decarboxylase system without the addition of ATP (6). This coenzyme also functions with the purified, cell-free tyrosine decarboxylase enzyme for which pyridoxal and ATP are inactive. The "natural" codecarboxylase from yeast is interchangeable with the coenzyme synthesized biologically or chemically from pyridoxal (6). The formation of coenzyme from pyridoxal by the action of chemical phosphorylating agents, as well as by the biological action of ATP, has been interpreted as evidence that the coenzyme is a phosphorylated derivative of pyridoxal. However, in the absence of chemical proof of structure and in view of the report of Gale and Epps (4) that the coenzyme recovered from yeast lacks phosphorus, this can be considered only as a hypothesis based upon these data. Because codecarboxylase is defined by its biological activity as the coenzyme of tyrosine (and lysine) decarboxylase, this term will at present serve both for the materials prepared from pyridoxal and for those recovered from yeast. The term does not denote the chemical structure of the coenzyme, which is still unknown; yet the preparation of codecarboxylase from pyridoxal does indicate that it is a derivative of pyridoxal. The biological data provide evidence for the similarity of the natural and synthetic coenzymes (6).

In view of the function of a member of the vitamin B_6 group as the coenzyme of tyrosine decarboxylase (3, 6), and the possibility that the tyrosine decarboxylase coenzyme recovered by Gale and Epps (4) and shown to function as the coenzyme of lysine decarboxylase is also a pyridoxal derivative, other amino acid decarboxylases have been studied to determine the possible rôle of this coenzyme. The data reported in this paper show that codecarboxylase also functions on the coenzyme of arginine and glutamic acid decarboxylases.

Methods

Bacterial cells were grown under conditions which favor the development of amino acid decarboxylases and dried *in vacuo* to yield cell preparations. The decarboxylase enzymes were separated from the cell preparations by autolysis and resolved by aging and dialysis to yield apoenzymes. Preparations of codecarboxylase from various sources were then studied to determine their ability to reactivate the apoenzyme.

The decarboxylase activity of the cells and cell-free enzymes was measured by conventional Warburg methods. Acetate or phthalate buffers were used between pH 4 and 5.5. Over this range the CO_2 is released as formed, so that the decarboxylase activity can be followed directly.

The activity of the various enzyme preparations is expressed in terms of an enzyme unit for purposes of comparison. 1 unit of decarboxylase is defined as that amount of enzyme which will liberate 100 microliters of CO_2 from the amino acid in question in 10 minutes under the conditions of the test; *i.e.*, at the optimum pH for each enzyme. All Warburg experiments were run at 28°. The enzyme activities are also expressed in Q_{CO_2} (protein), to indicate the purity of the preparations. Protein was determined by the biuret test of Robinson and Hogden (7).

Two principal sources of coenzyme were used: first, the "natural" codecarboxylase isolated from dried brewers' yeast by the procedure of Gale and Epps (4) and purified through "Stage 2;" second, "synthetic" codecarboxylase prepared from pyridoxal by treatment with $POCl_3$ (6). The quantity of coenzyme in both the natural and the synthetic preparations was determined by the assay method of Umbreit, Bellamy, and Gunsalus (6). This employs tyrosine decarboxylase apoenzyme obtained from cells grown in a medium deficient in members of the vitamin B_6 group (8). Coenzyme activities are expressed in terms of the amount of pyridoxal which, in the presence of ATP, will give the same rate of CO_2 evolution in the assay system; *i.e.*, if 1 ml. of codecarboxylase solution would activate the assay system to the same extent as 0.3 γ of pyridoxal (plus ATP), the codecarboxylase solution would be said to contain 0.3 γ of codecarboxylase. The actual weight of the codecarboxylase necessary to give this response is unknown and may be somewhat less than 0.3 γ in the example above. However, as pyridoxal is the only active material available in pure form, activities are related to it. When the codecarboxylase is available in pure form and its activity per unit weight is known, the actual amount of codecarboxylase in the solutions can be determined from the relation to pyridoxal quoted here.

Arginine Decarboxylase

Arginine decarboxylase is formed by a number of types of bacteria during growth under acid conditions (9). This enzyme acts on arginine to yield

the corresponding amine, agmatine, and carbon dioxide. Gale (10), in a review article, has reported the isolation of the enzyme in cell-free state from *Escherichia coli*, but further details are not available. However, from the later papers of Gale and Epps (5) and Epps (11) it appears that a number of the amino acid decarboxylases are globulins with sufficiently similar properties to render their separation difficult.

The present work involves the recovery of arginine decarboxylase from vacuum-dried *Escherichia coli* cells by the autolysis procedure of Gale and Epps (5), followed by purification to yield a water-clear enzyme solution. The apoenzyme was sufficiently resolved in these preparations to reveal the nature of the coenzyme.

Culture and Growth Conditions—By a proper selection of strains one may obtain a culture of *Escherichia coli* which possesses only one or perhaps two amino acid decarboxylases. For this study the "Crookes" strain (American Type Culture Collection, No. 8739) was used, because it possesses very active arginine and glutamic acid decarboxylases and is almost devoid of the lysine and tyrosine systems. Thus, any uncertainty which might arise from the presence of the coenzyme for the latter enzymes was minimized.

A very active arginine decarboxylase can be obtained by growing the Crookes strain of *Escherichia coli* in a broth composed of 1 per cent each of pepticase (Sheffield brand enzyme-hydrolyzed casein), yeast extract, and cerelose, and 0.5 per cent K_2HPO_4. In addition, the activity can be increased still further if 1 to 2 per cent pepticase is added after 12 to 15 hours incubation and the culture allowed to stand another 4 to 5 hours before the cells are harvested. The arginine decarboxylase used in this study was obtained from cells which were grown 15 hours at 25° in the above medium, followed by the aseptic addition of another 1 per cent pepticase and 0.5 per cent cerelose and a further 5 hour incubation. The cells were then harvested with a Sharples centrifuge, washed once with saline, suspended in distilled water, and dried *in vacuo* over drierite.

The resulting dried cell preparation decarboxylated several amino acids, as shown in Table I. The cell preparations were kept in the dry state over drierite, and were weighed just before use and suspended in saline. As Table I shows, arginine and glutamic acid decarboxylases were present in good yield. The traces of histidine, lysine, and tyrosine decarboxylases disappeared completely upon treatment of the dried preparations to isolate the decarboxylases.

Enzyme Purification and Resolution—As indicated in Table I, the dried cell preparation contained an arginine decarboxylase which was very active at pH 5.5. The Q_{CO_2} of 777 is equivalent to 1.3 enzyme units per mg. or, assuming that the cells contain 50 to 60 per cent protein, a Q_{CO_2} (pro-

tein) of around 1300. A cell-free enzyme was obtained from this preparation by the procedure of Gale and Epps (5). This involved autolysis for 2 hours at 37° in 0.15 M borate buffer at pH 8.2, followed by centrifugation to remove the cell débris. The resulting solution, which was turbid, contained approximately 40 per cent of the enzyme present in the cells before treatment. This solution had an arginine decarboxylase content expressed as Q_{CO_2} (protein) of 188 (Table II). The other 60 per cent of the enzyme activity was not present in the cell débris, being apparently destroyed during the autolysis. If the cell-free extract was allowed to stand in the refrigerator for about a week, part of the protein precipitated and could be removed by centrifugation. The resulting solution contained most of the enzyme, and, as inert protein had been precipitated, showed an increase of about 10-fold in the Q_{CO_2} (protein). Partial resolution of the enzyme also occurred during the process.

TABLE I

Amino Acid Decarboxylation by Dried Cell Preparations of Escherichia coli (Crookes)

The Warburg cups contain, in the side arm, 0.5 ml. of a M/15 solution of the amino acid to be studied; in the main compartment, 1 ml. of appropriate buffer (0.2 M acetate), 1 mg. of preparation, and water to make 2.5 ml. Temperature, 28°.

Amino acid	Q_{CO_2}	
	pH 5.5	pH 3.8
Arginine	777	62
Glutamic acid	280	18
Lysine	17	13
Tyrosine	5	9
Histidine	45	10

Adsorption procedures were of some value in the purification of the enzyme. In small scale experiments, the enzyme was almost completely adsorbed by tricalcium phosphate, Decalso, or Super Filtrol at pH 8.2. However, in experiments with larger amounts of enzyme only 20 to 30 per cent was adsorbed. Of this, about 70 per cent was loosely bound and could be eluted with distilled water, whereas the remaining 30 per cent was eluted only with 10 per cent KCl. Both the supernatant solutions and the eluates from Super Filtrol were fractionated further to yield water-clear enzyme preparations (Table II).

The purest arginine decarboxylase so far obtained still contained the glutamic acid decarboxylase in about the same proportion as was present in the intact cells. Attempts to remove the glutamic acid enzyme from the arginine preparation by adsorption were unsuccessful. Although such a separation should be possible, it may prove difficult because of the general similarity of the amino acid decarboxylase proteins (Gale and Epps (5)).

The arginine decarboxylase possesses a coenzyme which is partially removed during isolation and purification of the enzyme. This is readily seen from the data in Table II; *i.e.*, a 2- to 10-fold increase in the rate of arginine decarboxylation occurs upon addition of 0.8 γ of synthetic codecarboxylase to the purified enzyme solutions. Aging the enzyme preparations in the refrigerator at 0–5° is an effective method of resolution. The gradual loss of coenzyme in the refrigerator is apparently due to its destruction rather than to dissociation. A similar behavior was noted with the tyrosine decarboxylase of *Streptococcus faecalis* (6).

TABLE II

Cell-Free Arginine Decarboxylase from Escherichia coli (Crookes)

Per Warburg cup, in the side arm, 0.5 ml. of M/15 arginine, pH 5.5; in the cup, 1.0 ml. of 0.2 M acetate buffer, pH 5.5, cells or enzyme, and water or other additions to 2.5 ml.

Treatment	Character of extract	Recovery		Q_{CO_2} (protein)	
				Alone	Plus 0.8 γ coenzyme
		units	*per cent*		
700 mg. dried cell preparation		1000	100		
Cell-free extract	Turbid	363	36	188	188
30 units cell-free extract; exposed to air, 0°, pH 8, for 24 hrs	"	33	110	166	206
Same exposed 48 hrs	"	24	80	170*	212
90 units cell-free extract; adsorbed with Super Filtrol (Supernatant 1)	"	74	83		149
50 units Supernatant 1; exposed to air, 0°, pH 8, 24 hrs	"	52	108	122	165
Same then held in closed tube 7 days and centrifuged (Supernatant 2)	Clear†	16	30	870	1010
25 units Supernatant 1; held in closed tube, 0°, 7 days; centrifuged (Supernatant 3)	"	16	64	650	1270
364 units cell-free extract; adsorbed with Super Filtrol; 108 units adsorbed; eluted with distilled water	Faintly turbid	78	21	1600	2400
Eluate stored, 0°, 8 days	" "	24	32	78	642

* Pyridoxal, or pyridoxal plus adenosine triphosphate, shows no stimulation.

† The glutamic decarboxylase is still present, Q_{CO_2} (protein) 145, with coenzyme, 206.

The nature of the coenzyme is also evident from these data. The coenzyme used for stimulation of the partially resolved enzyme (Table II) was synthetic codecarboxylase obtained by treating pyridoxal with $POCl_3$ by various modifications of Zeile and Fawaz's procedure (12) for the syn-

thesis of phosphocreatine. The same preparation was not used throughout, but in each case the codecarboxylase content of the material was determined with the tyrosine assay system (6).

Thus synthetic codecarboxylase prepared from pyridoxal and shown to act as the coenzyme of tyrosine decarboxylase also functions as the coenzyme of arginine decarboxylase.

The cell-free tyrosine decarboxylase will convert pyridoxal to the coenzyme if ATP is also supplied (6); thus, one would expect that the necessary auxiliary enzymes for this conversion might also be present in the cell-free extracts of *Escherichia coli*. However, this is not the case, for the addition of pyridoxal (with or without ATP) did not result in increased arginine decarboxylation (see foot-note, Table II).

The "natural" codecarboxylase preparations from dried brewers' yeast, purified through Stage 2 of the procedure of Gale and Epps (4), stimulated the partially resolved arginine decarboxylase as well as did the "synthetic" codecarboxylase. Inasmuch as codecarboxylase from two entirely independent sources will activate the arginine decarboxylase, it is concluded that the coenzyme for arginine decarboxylase is the same as that for the tyrosine and lysine decarboxylases.

A third line of evidence of the nature of the coenzyme of the arginine decarboxylase is also available. That is, the partially purified enzyme solutions were heated to destroy the enzyme, and the coenzyme was extracted and tested for its ability to activate the tyrosine system in the assay procedure for codecarboxylase (6). Assay of the two water-clear arginine preparations (Table II) showed 0.96 γ of codecarboxylase per unit of enzyme present in the first sample and 1.1 γ per unit in the second; thus the codecarboxylase content, as estimated by tyrosine decarboxylation, is approximately proportional to the arginine decarboxylating activity.

Dialysis was used in an attempt to resolve the enzyme but was ineffective, as was the case with the lysine and tyrosine systems. Prolonged dialysis coupled with aging will effect some separation. For example, dialysis for 48 hours against 500 volumes of 0.001 M acetate at pH 5.5 resulted in a preparation with a Q_{CO_2} (protein) of 400 which was stimulated by codecarboxylase to Q_{CO_2} 810 (*i.e.*, about 50 per cent resolution). The enzyme is irreversibly inactivated by dialysis at pH 8.

Glutamic Acid Decarboxylase

As noted previously, this enzyme was associated with the arginine decarboxylase and can be obtained in a water-clear cell-free state (*cf.* Table II). Also the glutamic acid decarboxylase can be resolved into the apoenzyme which is stimulated by the synthetic coenzyme active for tyrosine, lysine,

or arginine decarboxylase. There is, therefore, presumptive evidence that pyridoxal in the form of codecarboxylase also functions as the coenzyme of glutamic acid decarboxylase.

The glutamic acid decarboxylase, which is widely distributed in microorganisms (9), liberates 1 mole of CO_2 from glutamic acid to yield γ-aminobutyric acid. The optimum pH for activity of the enzyme with intact cells is pH 4 (9), but the cell-free enzyme has an optimum closer to pH 5. The "Crookes" strain of *Escherichia coli* contained a very active glutamic acid decarboxylase (Table I) but as the arginine enzyme was present in even greater concentration another strain was sought as a source of this enzyme free of the other decarboxylases. The American Type Culture Collection strain 4157 was found to lack the arginine enzyme and to contain only the lysine enzyme, among those tested, in addition to the glutamic acid decarboxylase. Strain 4157 is listed as National Collection of Type Cultures (London) No. 86. The latter is one of the strains employed by Gale (9) and found to contain a number of decarboxylases, but to lack the glutamic system. An explanation for this difference is not available; it is possible that strains have been mixed in stock collections.

The glutamic enzyme was obtained in fair activity by growing the culture as follows: 10 liters of a medium composed of 1 per cent pepticase, 0.1 per cent yeast extract, 0.25 per cent K_2HPO_4, and 1 per cent cerelose were inoculated with 40 ml. of an 8 hour culture of *Escherichia coli*, strain 4157, and incubated at 25°. After 15 hours maximum growth was obtained and the pH had fallen to 4.7; the culture at this stage possessed the glutamic acid and the lysine decarboxylases with Q_{CO_2} of 53 and 120 respectively. The culture was allowed to incubate an additional 24 hours, and the cells harvested by Sharples centrifuge, washed with saline, suspended in distilled water, and dried over drierite. The resulting dried cell preparation possessed only the glutamic acid decarboxylase; the activity for lysine was apparently destroyed during the drying process. When tested at pH 5.0, the Q_{CO_2} (glutamic) was 43.5, while the decarboxylases for tyrosine, lysine, arginine, and histidine were completely lacking.

The cell-free glutamic acid decarboxylase was obtained from these cells by the autolysis procedure of Gale and Epps (5). In this case 500 mg. of dry preparation were incubated 2 hours at 37°, in 0.02 M phosphate buffer of pH 7.0, and the cell débris removed by centrifugation. The resulting cell-free extract was turbid, but contained a reasonably active glutamic acid decarboxylase (Table III). Considerable loss of the enzyme occurred during the extraction procedure, and further loss resulted from the process of resolution. In this case, dialysis was reasonably effective in the removal of the coenzyme, as shown in Table III.

Those preparations from which part of the coenzyme had been removed

by dialysis, or by aging, could be reactivated by the "natural" coenzyme or, when tested, by the "synthetic" coenzyme, Table III. Pyridoxal, plus ATP, also restored the activity of the enzyme preparations. Therefore, the enzymes necessary for the conversion of the pyridoxal into coenzyme are present, as was the case with the tyrosine preparations (6).

Thus the coenzyme of glutamic acid decarboxylase is also a pyridoxal derivative. If the natural and synthetic codecarboxylases are similar, as is indicated by their equivalence in the tyrosine (6), arginine, and glutamic acid decarboxylase systems, one may conclude that the "natural" codecarboxylase is also a pyridoxal derivative and thus that the coenzyme

TABLE III

Cell-Free Glutamic Acid Decarboxylase from Escherichia coli (Strain 4157)

Per Warburg cup, in the side arm, 0.5 ml. of M/15 glutamic acid, pH 5.0; in the cup, 1.0 ml. of 0.07 M phthalate, pH 5.0, cells or enzyme, and other additions to 2.5 ml.

Treatment	Character of extract	Recovery		Q_{CO_2} (protein)		
				Alone	Plus 0.6 γ coenzyme*	Plus 6 γ pyridoxal + 1 mg. adenosine triphosphate
		units	*per cent*			
500 mg. dried cell preparation...		360	100			
Cell-free extract................	Turbid	46	13	87	96	110†
46 units cell-free extract; incubated 8 days, 0°, pH 7.0, centrifuged (Supernatant 1)......	Almost clear	5	11	210	295	
43 units cell-free extract; dialyzed 24 hrs., 0°, pH 4.5......	Faintly turbid	2.4	5	52	110	123
43 units cell-free extract; dialyzed, 0°, pH 2...............	" "	5.2	12	163	260	302

* Natural coenzyme from yeast (Gale and Epps); see "Methods."

† Synthetic coenzyme, 0.8 γ.

of lysine decarboxylase is a member of the vitamin B_6 group. In view of these data it seems probable that one of the functions of the vitamin B_6 group is to act as the general coenzyme of amino acid decarboxylases.

SUMMARY

Arginine decarboxylase and glutamic acid decarboxylase have been isolated in cell-free state from dried preparations of *Escherichia coli*, and each has been shown to require a coenzyme for activity.

Both enzymes require the codecarboxylase which has previously been identified as the coenzyme of the lysine and tyrosine decarboxylases.

The synthetic codecarboxylase prepared from pyridoxal has been shown to be active with each enzyme.

From the fact that codecarboxylase has been shown to function as the coenzyme of four amino acid decarboxylases, and synthetic codecarboxylase prepared from pyridoxal has been shown to function in three of these, it is suggested that one of the functions of the vitamin B_6 group is as the general coenzyme of amino acid decarboxylases.

BIBLIOGRAPHY

1. Snell, E. E., *J. Biol. Chem.*, **154**, 313 (1944).
2. Gunsalus, I. C., and Bellamy, W. D., *J. Biol. Chem.*, **155**, 357 (1944).
3. Gunsalus, I. C., Bellamy, W. D., and Umbreit, W. W., *J. Biol. Chem.*, **155**, 685 (1944).
4. Gale, E. F., and Epps, H. M. R., *Biochem. J.*, **38**, 250 (1944).
5. Gale, E. F., and Epps, H. M. R., *Biochem. J.*, **38**, 232 (1944).
6. Umbreit, W. W., Bellamy, W. D., and Gunsalus, I. C., *Arch. Biochem.*, in press.
7. Robinson, H. W., and Hogden, C. G., *J. Biol. Chem.*, **135**, 707 (1940).
8. Bellamy, W. D., and Gunsalus, I. C., *J. Bact.*, in press.
9. Gale, E. F., *Biochem. J.*, **34**, 392 (1940).
10. Gale, E. F., *Bact. Rev.*, **7**, 137 (1943).
11. Epps, H. M. R., *Biochem. J.*, **38**, 242 (1944).
12. Zeile, K., and Fawaz, G., *Z. physiol. Chem.*, **256**, 193 (1938).

THE ENZYMATIC SYNTHESIS OF PHOSPHOPYRUVATE FROM PYRUVATE

By HENRY A. LARDY* and JOHN A. ZIEGLER†

(From the Departments of Chemistry and Pathological Chemistry, Banting Institute, University of Toronto, Toronto, Canada)

(Received for publication, March 22, 1945)

In the pathway of glycogen synthesis from pyruvate and lactate, one of the steps in the concatenated reactions known as the Embden-Meyerhof scheme which has remained obscure is the mechanism of phosphopyruvate "resynthesis." This problem has been the subject of much speculation and some investigation. Since Meyerhof, Ohlmeyer, Gentner, and Maier-Leibnitz (1) reported the reaction,

(1) Phosphopyruvate + ADP → pyruvate + ATP

to be irreversible, a number of alternate pathways have been proposed for the conversion of pyruvate, lactate, and other carbohydrate fragments to phosphopyruvate.

In accordance with Kalckar's demonstration of phosphopyruvate (hereafter ~phPy)[1] synthesis during the oxidation of fumarate by kidney extracts (3), it was suggested that this enol phosphate arose by a phosphorylation directly coupled with the oxidation of fumarate (Lipmann (2)). The finding that about one-sixth of the newly deposited glycogen, following lactate, pyruvate, or glucose feeding to rats, originated from tissue bicarbonate led Solomon, Vennesland, Klemperer, Buchanan, and Hastings (4) to postulate that the formation of a 4-carbon compound as a precursor of ~phPy is a logical step in glycogen synthesis from pyruvate. Ferdman and Epstein (5) reported ~phPy synthesis during the oxidation of lactate by cat muscle. In view of the fact that their medium contained bicarbonate, it is possible that oxalacetate may have been formed and that subsequent phosphorylation of the 4-carbon acid could have led to ~phPy formation.

Leloir and Muñoz (6) found liver preparations to produce ~phPy during the oxidation of succinate, fumarate, and citrate but not of lactate and pyruvate. Other data presented by these workers have been previously interpreted (7) as indicating that ~phPy may not have arisen by direct phosphorylation of a 4-carbon compound.

* National Research Council (Washington) Fellow in Chemistry 1944–45.

† Aided by a grant from the National Research Council of Canada.

[1] Other abbreviations used in this paper are ATP for adenosine triphosphate, ADP for adenosine diphosphate, ~ph for high energy phosphate (2), ~phCr for phosphocreatine.

In a previous attempt to demonstrate the formation of phosphoenoloxalacetate it was found that addition of oxalacetate, or pyruvate plus bicarbonate, to aged rat muscle extracts resulted in a loss of ATP and an increase in inorganic phosphate but no accumulation of enol phosphate could be detected (8).

The experiment on which the Heidelberg workers (1) based their conclusions regarding the irreversibility of Reaction 1 was designed to detect any exchange of ~ph between ATP and ~phPy and did not depend on an accumulation of ~phPy produced from pyruvate and ATP. The group potential of the enol phosphate has been calculated by Lipmann ((2) p. 110), from thermal data, to be approximately 11,250 calories, or of the same magnitude as that of the two terminal phosphates of ATP. From a thermodynamic point of view, therefore, it seems that, in the steady state, phosphate exchange should occur between ATP and ~phPy. Meyerhof apparently recognized this, for, in discussing the experiment from which it was concluded that Reaction 1 is irreversible, he stated in 1941 (9), "But we must concede that the experimental basis for this negative result is not too large and therefore accept it with some reservation until it is more firmly established."

The discovery, by Boyer *et al.* (10, 11), that K^+ is necessary for the transfer of ~ph from ~phPy to the adenylic system bears strongly on the validity of that conclusion, for the Heidelberg workers employed an enzyme extract which has been dialyzed for 12 hours and the protocol of their experiment does not indicate the addition of any potassium salt. The reaction has therefore been investigated with radioactive phosphorus, and the relation of various cations to the reversal of Reaction 1 and to the oxidative phosphorylation of *d*-glyceraldehyde 3-phosphate has been studied.

Methods

All experiments reported in this paper were made with a dialyzed extract of acetone-precipitated rat muscle extract prepared as described elsewhere (11). *dl*-Glyceraldehyde 3-phosphate (Fischer-Baer ester) was synthesized according to the new procedure of Baer and Fischer (12); the other substrates were as previously described (10, 11).[2] All acids were neutralized with sodium hydroxide and the final pH of the reaction mixtures was always 7.4. No bicarbonate was added in any of the experiments.

The enzyme reaction mixtures were deproteinized with trichloroacetic acid and immediately placed in an ice-salt bath. Inorganic phosphate

[2] The cozymase used in these experiments was kindly supplied by Dr. D. F. Green of Merck and Company, Inc., Rahway, New Jersey, the desoxycorticosterone acetate by Dr. Erwin Schwenk of the Schering Corporation, Bloomfield, New Jersey.

was separated from the filtrate as $MgNH_4PO_4 \cdot 6H_2O$ by treatment with magnesia mixture (13). ~phPy was then hydrolyzed by the alkaline iodine procedure of Lohmann and Meyerhof (14).

Following removal of the excess iodine under acid conditions the inorganic phosphate liberated was either separated for determination of radioactivity by treatment with magnesia mixture or, in other experiments, was determined quantitatively after removal of the iodoform. The labile phosphate groups of adenosine polyphosphates were hydrolyzed by 1 N HCl for 7 minutes at 100° and the inorganic phosphate was separated with magnesia mixture. The inorganic phosphate fractions were analyzed by a micro modification of the Fiske and Subbarow method (15) adapted to a photoelectric colorimeter.

The radioactivity of aliquots of the phosphate fractions was determined with a Lauritsen electroscope.[3] The activity, measured as net rate of deflection, is expressed as divisions of deflection per minute per mg. of phosphorus.

~phCr was determined in the fraction of the filtrate soluble in 2 per cent $CaCl_2$ at pH 8.2 (16). The alkali lability of triose phosphate (17) interferes with the accurate determination of both ~phCr and ~phPy and therefore the ester is converted to inorganic phosphate before proceeding with the fractionations. This was accomplished by making the filtrates containing triose phosphate 1 N with NaOH and holding for 30 minutes at room temperature.

Results

Preliminary experiments with an undialyzed extract of muscle acetone powder demonstrated that inorganic phosphate containing P^{32} was incorporated into the labile phosphate of ATP during the oxidation of *d*-glyceraldehyde 3-phosphate and that this labeled ~ph was in turn introduced into ~phPy. Since K^+ is necessary to catalyze Reaction 1 (10, 11) and in view of the fact that the Heidelberg workers used a dialyzed enzyme and added no potassium salts to their reaction mixture, it seemed reasonable that the distribution of ~ph between ATP and ~phPy was contingent on the presence of K^+. The results of experiments testing this possibility are shown in Table I. The ~phPy in these experiments was produced enzymatically (18) by incubating the phosphoglycerate and enzyme for 10 minutes at 25°, followed by 10 minutes at 37° before the addition of fluoride. Experimental incubation time was measured from the addition of ATP to deproteinization.

The traces of radioactivity appearing in the ~phPy fraction at zero

[3] We are indebted to Mr. D. Scott of the Banting and Best Department of Medical Research for the radioactivity measurements.

time are the result of incomplete precipitation of inorganic phosphate by the first treatment with magnesia mixture. In five other zero time samples the degree of contamination of the ~phPy fraction never exceeded that obtained in Experiments A and B. The decrease in activity of the inorganic fraction during incubation is the result of P^{32} transfer to other fractions and of the increase in inorganic phosphate due to spontaneous decomposition of 1,3-diphosphoglycerate (19, 20).

The equilibrium between ~phPy and ATP, which in these experiments was followed by the distribution of P^{32}, can be seen to be hastened by the

TABLE I

Exchange of High Energy Phosphate between Phosphopyruvate and Adenosine Triphosphate

At the time of fluoride addition each tube contained the following (expressed in micromoles): phosphoglycerate 80, phosphopyruvate 20, lactate 100, $MgCl_2$ 20, $MnSO_4$ 8, cozymase 0.3, inorganic phosphate (containing P^{32}) 33, and 1.6 ml. of enzyme solution. After the addition of 200 micromoles of NaF, 3.2 micromoles of adenosine triphosphate were added, giving a final volume of 4 ml. Incubated 15 minutes at 37°.

Experiment	KCl added	P^{32} content of phosphate fractions expressed in divisions per min. per mg. P		
		Inorganic	Adenosine triphosphate	Phosphopyruvate
	micromoles			
Zero time	200	62	0.0	1.3
A	None	46	15	5.7
	200	42	15	8.6
Zero time	None	63	1.0	1.4
B	"	53	15	6.3
	200	47	14	11
	200*	49	13	8.4

* 2 mg. of desoxycorticosterone acetate were added.

addition of K^+. The incorporation of P^{32} into the various fractions is the result of the following equilibrium reactions.

(2) *d*-Glyceraldehyde 3-phosphate + inorganic phosphate + cozymase $\rightleftharpoons$ 1,3-diphosphoglycerate + reduced cozymase

(3) Reduced cozymase + pyruvate $\rightleftharpoons$ cozymase + lactate

(4) 1,3-Diphosphoglycerate + ADP $\rightleftharpoons$ 3-phosphoglycerate + ATP

(5) Pyruvate + ATP $\underset{}{\overset{(K^+)}{\rightleftharpoons}}$ phosphopyruvate + ADP

Phosphate exchange between ~phPy and ATP occurred to an appreciable extent even without added potassium but, as shown in Table II, the

enzyme was only partially depleted of K^+ by the 48 hour dialysis. The enzyme solution must have retained sufficient quantities of K^+ to catalyze the transfer of phosphate from ~phPy to ADP but at a rate *not greater than* three-sevenths that obtained with added potassium.

K^+, Mg^{++}, and Mn^{++} in Relation to Phosphorylations Coupled with Oxidation of d-Glyceraldehyde 3-Phosphate—From the above results it appeared that the incorporation of inorganic phosphate into ATP (through the

TABLE II

Extent of Dialysis of Enzyme and Proof of Fluoride Inhibition

Each tube contained the following additions (expressed in micromoles) in a final volume of 1 ml.: phosphoglycerate 10, creatine 50, adenosine triphosphate 0.16, inorganic phosphate 100, and 0.5 ml. of enzyme solution. Incubated 10 minutes at 37°.

Other additions			Phosphocreatine synthesized
$MgCl_2$	KCl	NaF	
micromoles	*micromoles*	*micromoles*	*micromoles*
	100		0.1
5			1.2
5	100		2.8
5	100	50	0.1

TABLE III

Phosphorylation of Creatine during Oxidation of Fischer-Baer Ester

Each tube contained the following additions (expressed in micromoles) in a final volume of 2 ml.: inorganic phosphate 100, *dl*-glyceraldehyde 3-phosphate 30 (of *d* component), cozymase 0.15, pyruvate 200, creatine 80, adenosine triphosphate 0.16, $MgCl_2$ 20, $MnSO_4$ 3, NaF 120, and 0.7 ml. of enzyme solution. Incubated 20 minutes at 37°.

KCl added	Phosphocreatine synthesized
micromoles	*micromoles*
None	8.3
200	7.4

oxidation of glyceraldehyde phosphate) was influenced less by potassium than the equilibrium between ~phPy and ATP. The effect of K^+, Mg^{++}, and Mn^{++} on the oxidative phosphorylation was therefore investigated. The results in Table III show that added K^+ was not required for Reactions 2, 3, and 4. It was shown by Boyer *et al.* (10) that K^+ is not required for the phosphorylation of creatine by ATP; since Mg^{++} is required for the latter phosphorylation (21), its requirement for Reactions 2, 3, and 4 could not be tested by the system shown in Table III.

In experiments with adenylic acid as ~ph acceptor, it was found (Table IV) that Mg^{++} or Mn^{++} additions were also unnecessary for the oxidation of glyceraldehyde phosphate and the transfer of ~ph from 1,3-diphosphoglycerate to the adenylic system. The addition of traces of ATP was not required to initiate the phosphorylation of adenylic acid. In the phosphorylation of creatine by ~phPy, adenylic acid is not nearly as effective a ~ph carrier as the higher phosphorylated adenylic compounds (21, 11) but where there is an accumulation of ~ph in the adenylic system, as in Table IV or in the phosphorylation of adenylic acid by ~phPy (other experiments), additions of ADP or ATP do not accelerate the process.

TABLE IV

Fixation of Inorganic Phosphate during Oxidation of Fischer-Baer Ester and Transfer of Phosphate to Adenylic Acid

Each tube contained the following additions (expressed in micromoles) in a final volume of 2 ml.: inorganic phosphate 20, *dl*-glyceraldehyde 3-phosphate 20 (of *d* component), cozymase 0.15, pyruvate 200, adenylic acid 10 (capable of accepting 20 micromoles of high energy phosphate), NaF 100, and 0.7 ml. of enzyme solution. Incubated 8 minutes at 37°.

Other additions			Inorganic phosphate taken up
$MgCl_2$	$MnSO_4$	Adenosine triphosphate	
micromoles	*micromoles*	*micromole*	*micromoles*
		0.16	4.0
10		0.16	4.1
	10	0.16	5.4
10	10	0.16	4.0
10	10		4.0

TABLE V

Synthesis of Phosphopyruvate during Oxidation of Fischer-Baer Ester

Each tube contained the following additions (expressed in micromoles) in a final volume of 2.25 ml.: inorganic phosphate 150, pyruvate 300, *dl*-glyceraldehyde 3-phosphate 20 (of *d* component), cozymase 0.15, hexose diphosphate 20, NaF 120, adenosine triphosphate 0.4, $MgCl_2$ 20, $MnSO_4$ 10, and 0.63 ml. of enzyme solution. Incubated at 37°.

Experiment	Time of incubation	KCl added	Phosphopyruvate synthesized
	min.	*micromoles*	*micromole*
A	20	None	0.00
		200	0.58
B	12	None	0.06
		200	0.52

Synthesis of Phosphopyruvate—Since Reaction 5 was shown in the above experiments with P^{32} to be reversible, the synthesis of ~phPy from pyruvate should occur when the supply of ~ph is continually being renewed by another reaction. As shown in Table V, ~phPy was synthesized from pyruvate when the ~ph was produced by oxidation of glyceraldehyde 3-phosphate, but *only when* K^+ *was added.* The ~phPy values for each sample are corrected for traces of inorganic phosphate remaining after the treatment with magnesia mixture. Although the amounts of ~phPy synthesized are small, they are far greater than the limit of accuracy of the analytical methods (0.05 micromole) and it must be remembered that the 3-phosphoglycerate produced also competes with pyruvate for the ~ph. The 1,3-diphosphoglycerate produced in the competitive reaction spontaneously decomposes (19, 20) to give inorganic phosphate and 3-phosphoglycerate which may again accept ~ph. Hexose diphosphate was added in these experiments to prevent removal of *d*-glyceraldehyde 3-phosphate[4] by the isomerase-aldolase equilibrium.

The concentration of NaF in these experiments is far greater than that required completely to inhibit enolase (23) (see also Table II). Further evidence that the ~phPy produced did not come from phosphoglycerate is the fact that ~phPy appeared only when K^+ was added.

DISCUSSION

It has been experimentally demonstrated by the distribution of P^{32} and by direct synthesis that pyruvate may be enzymatically phosphorylated by ATP in the presence of K^+ and Mg^{++} to produce ~phPy. The implications of this finding for the mechanism of glycogen synthesis are evident. It is no longer necessary to postulate the occurrence of a 4-carbon intermediate in the synthesis of ~phPy from pyruvate. It is a thermodynamic fact that a pathway of ~phPy synthesis from pyruvate through a 4-carbon intermediate, regardless of its mechanism, can be energetically no more economical than the direct synthesis by Reaction 5. In the intact organism the continually applied "potential" of ~ph produced by the oxidation of carbohydrate (see (2) and (24)) or fat (25) can supply the energy for the synthesis of hexoses from pyruvate. The finding of Buchanan, Hastings, and Nesbett (26) that high concentrations of K^+ were necessary to obtain glycogen formation from pyruvate in liver slices can be explained, at least in part, by the necessity of K^+ for Reaction 5.

[4] Only the *d* component of the Fischer-Baer ester condenses in the presence of rat muscle isomerase and aldolase at low temperature to produce hexose diphosphate (H. A. L., unpublished data). *l*-Glyceraldehyde 3-phosphate differs from the free *l*-glyceraldehyde in that the latter can condense with dihydroxyacetone phosphate to form *l*-sorbose 1-phosphate (22).

The present findings do not rule out the possibility of ~phPy formation, by direct oxidative phosphorylation of a 4-carbon dicarboxylic acid, but they do eliminate such a 4-carbon compound as an *obligatory* intermediate in the conversion of pyruvate to ~phPy.

The mechanism by which bicarbonate is incorporated into glycogen will probably be disclosed by further studies on the reversibility of pyruvate decarboxylation (27, 28) and on the extent to which the enol group of oxalacetate can shift between carbon atoms 2 and 3 (29, 30).

SUMMARY

It has been demonstrated by two separate enzymatic techniques that the reaction

$$\text{Pyruvate} + \text{ATP} \rightleftharpoons \text{phosphopyruvate} + \text{ADP}$$

is reversible.

P^{32} incorporated into ATP during the oxidation of glyceraldehyde 3-phosphate was in equilibrium with that in phosphopyruvate.

Pyruvate was enzymatically phosphorylated when high energy phosphate was continually supplied by the oxidation of glyceraldehyde 3-phosphate. The importance of K^+ for the synthesis of phosphopyruvate from pyruvate was demonstrated.

K^+ and Mg^{++} or Mn^{++} are needed in much lower concentrations (if at all) for the oxidation of glyceraldehyde 3-phosphate and the transfer of high energy phosphate to the adenylic system than for the transfer from phosphoglycerate through phosphopyruvate to adenylate.

I am indebted to Professors Hermann O. L. Fischer and Andrew Hunter, in whose laboratories these experiments were conducted, for kindly furnishing supplies and equipment, and to Professor Erich Baer who introduced to me the synthesis of *dl*-glyceraldehyde 3-phosphate. (H. A. L.)

BIBLIOGRAPHY

1. Meyerhof, O., Ohlmeyer, P., Gentner, W., and Maier-Leibnitz, H., *Biochem. Z.*, **298**, 396 (1938).
2. Lipmann, F., in Nord, F. F., and Werkman, C. H., Advances in enzymology and related subjects, New York, **1**, 99 (1941).
3. Kalckar, H., *Biochem. J.*, **33**, 631 (1939).
4. Solomon, A. K., Vennesland, B., Klemperer, F. W., Buchanan, J. M., and Hastings, A. B., *J. Biol. Chem.*, **140**, 171 (1941).
5. Ferdman, D., and Epstein, S., *Science*, **91**, 365 (1940).
6. Leloir, L. F., and Muñoz, J. M., *J. Biol. Chem.*, **153**, 53 (1944).
7. Lardy, H. A., and Elvehjem, C. A., in Annual review of biochemistry, Stanford University, **14**, 1 (1945).

8. Lardy, H. A., Boyer, P. D., and Phillips, P. H., *Arch. Biochem.*, **5**, 295 (1944).
9. Meyerhof, O., in A symposium on respiratory enzymes, Madison, 12 (1942).
10. Boyer, P. D., Lardy, H. A., and Phillips, P. H., *J. Biol. Chem.*, **146**, 673 (1942).
11. Boyer, P. D., Lardy, H. A., and Phillips, P. H., *J. Biol. Chem.*, **149**, 529 (1943).
12. Baer, E., and Fischer, H. O. L., *J. Biol. Chem.*, **150**, 223 (1943).
13. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric techniques and related methods for the study of tissue metabolism, Minneapolis (1945).
14. Lohmann, K., and Meyerhof, O., *Biochem. Z.*, **273**, 60 (1934).
15. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
16. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **81**, 629 (1929).
17. Fischer, H. O. L., and Baer, E., *Ber. chem. Ges.*, **65**, 337 (1932).
18. Meyerhof, O., and Kiessling, W., *Biochem. Z.*, **276**, 239 (1935).
19. Meyerhof, O., and Junowicz-Kocholaty, R., *J. Biol. Chem.*, **145**, 443 (1942).
20. Negelein, E., and Brömel, H., *Biochem. Z.*, **303**, 132 (1939).
21. Lehmann, H., *Biochem. Z.*, **281**, 271 (1935).
22. Meyerhof, O., Lohmann, K., and Schuster, P., *Biochem. Z.*, **286**, 319 (1936).
23. Lohmann, K., and Meyerhof, O., *Biochem. Z.*, **273**, 60 (1934).
24. Kalckar, H., *Chem. Rev.*, **28**, 71 (1941).
25. Lardy, H. A., Hansen, R. G., and Phillips, P. H., *Arch. Biochem.*, **6**, 41 (1945).
26. Buchanan, J. M., Hastings, A. B., and Nesbett, F. B., *J. Biol. Chem.*, **145**, 715 (1942).
27. Utter, M. F., Werkman, C. H., and Lipmann, F., *J. Biol. Chem.*, **154**, 723 (1944).
28. Lipmann, F., and Tuttle, L. C., *J. Biol. Chem.*, **154**, 725 (1944).
29. Evans, E. A., Jr., Vennesland, B., and Slotin, L., *J. Biol. Chem.*, **147**, 771 (1943).
30. Wood, H. G., Werkman, C. H., Hemingway, A., and Nier, A. O., *J. Biol. Chem.*, **142**, 31 (1942).

ISOLATION AND PROPERTIES OF THE ANTERIOR HYPOPHYSEAL GROWTH HORMONE*

By CHOH HAO LI, HERBERT M. EVANS, AND MIRIAM E. SIMPSON

(From the Institute of Experimental Biology, University of California, Berkeley)

(Received for publication, March 22, 1945)

During the last decade the question has been raised (1, 2) as to the existence of the anterior hypophyseal growth hormone. One of us (3) has answered this contention elsewhere but it should be conceded that final proof for the growth hormone as a separate entity can only be obtained by the isolation of the hormone freed from other active components as well as from inert proteins. In this paper we will describe a method, briefly reported earlier (4), for isolating a protein from the anterior lobe of ox pituitary, which can be shown to have the biological characteristics of the growth hormone.

Method of Assay

For the routine assay of growth hormone preparations hypophysectomized female rats, 26 to 28 days of age at the time of the operation and 10 to 14 days postoperative, were used. The body growth of these animals after nine intraperitoneal injections in 10 days was taken as the estimation of the growth-promoting activity of a preparation (5, 6).

Method of Isolation

The extraction of growth hormone from fresh anterior pituitary substance or acetone-dried glands is generally carried out in alkaline solutions (7–10). The methods published from this laboratory in recent years involve a treatment with cysteine (9) which destroys lactogenic, thyrotropic, and gonadotropic activities but not growth potency; while biological purification was achieved by this method, chemical purification was not obtained. The method herein described for the isolation of growth hormone does not include the cysteine step but involves salt fractionation and isoelectric precipitation.

1. Acetone-Dried Powder—The dissected anterior lobes of ox pituitaries are ground and dried with chilled acetone (−10°). From 1 kilo of anterior lobes of beef pituitaries the yield of dried powder is approximately 250 gm. This material can be stored indefinitely without loss of growth potency if it is completely dried.

2. Calcium Hydroxide Extract—The acetone-dried powder (400 gm.) is

* Aided by grants from the Rockefeller Foundation, New York; the Josiah Macy, Jr., Foundation, New York; and the National Research Council Committee on Research in Endocrinology.

extracted with 12 liters of $Ca(OH)_2$ solution of about pH 11.5 for 24 hours; the pH is then lowered to 8.7 by adding CO_2 gas. The insoluble material, which should settle easily within 24 or 48 hours, is removed by centrifugation. This step, as are all other steps, is carried out at 2–3°.

3. Globulin Fraction—The $Ca(OH)_2$ extract is brought to 2.0 M by the addition of solid $(NH_4)_2SO_4$. The resulting precipitate is suspended in 4 liters of water and the solution is brought to 0.6 M with $(NH_4)_2SO_4$. The precipitate is centrifuged and discarded. The supernatant is next precipitated again with 2.0 M $(NH_4)_2SO_4$. The precipitate formed is dissolved in 1 liter of water and dialyzed until salt-free. The pH of the thoroughly dialyzed material should be 6.5 to 7.0. If this pH range is not achieved, a few drops of 1 M HCl or NaOH may be added. The insoluble material in the dialysis sac contains most of the growth-stimulating protein and is called the "globulin fraction."

4. NaCl Fractionation—The globulin fraction is dissolved in 600 cc. of solution by the addition of 1.0 M HCl until the pH becomes 4.0. To this solution a saturated NaCl solution is added to a salt concentration of 0.10 M. The precipitate formed is devoid of growth activity and is therefore discarded. The supernatant is brought to 5.0 M with solid NaCl. The 5.0 M NaCl precipitate is redissolved in 300 cc. of solution of pH 4.0 and the NaCl fractionation is repeated twice.

5. pH and $(NH_4)_2SO_4$ Fractionation—The final 5.0 M NaCl precipitate is dissolved in 200 cc. of water and dialyzed until salt-free. The dialyzed solution is adjusted to pH 5.7 to 5.8 and the precipitate formed is centrifuged off. The supernatant is then made alkaline and adjusted to pH 8.7 to 8.8, the precipitate again being removed by centrifugation. The clear fluid is then next brought to 1.65 M with $(NH_4)_2SO_4$ at pH 7.0. The $(NH_4)_2SO_4$ precipitate is dissolved in 150 cc. of water and dialyzed. The change in pH and the $(NH_4)_2SO_4$ fractionation are repeated twice.

6. Isoelectric Precipitation—The dialyzed solution of the final 1.65 M $(NH_4)_2SO_4$ precipitate is precipitated at pH 5.7 to 5.8 and pH 8.7 to 8.8 as above and finally at pH 6.8 to 6.9 in the absence of $(NH_4)_2SO_4$. This isoelectric precipitation is repeated twice. The final precipitate at pH 6.8 to 6.9 is the growth hormone.

Table I presents the averaged protein yields of nitrogen in each step from twenty-five experiments. It may be noted that approximately 0.040 gm. of the growth hormone was isolated from 1 kilo of ox anterior pituitary substance.

Biological Potency

When growth hormone preparations obtained by the above procedure are assayed in hypophysectomized female rats (intraperitoneal injections, 4 days), a total dose of 5 mg. gives no histological evidence of the presence of

adrenocorticotropic, thyrotropic, or gonadotropic hormonal contaminations. In addition, when a total dose of 10 mg. was injected subcutaneously into month-old squabs (4 days), the absence of crop sac response indicates the absence of the lactogenic hormone.

TABLE I

Yield of Protein Nitrogen in Isolation Procedure with 400 Gm of Acetone-Dried Ox Anterior Pituitary As Starting Material

Isolation step No (see the text)	Fraction	Yield of protein N
		mg
2	$Ca(OH)_2$ extract	7000
3 and 4	Solution at pH 4 0 of "globulin fraction"	1300
4	First 0 10 M NaCl-soluble material	500
5	Dialyzed material of final 1 65 M $(NH_4)_2SO_4$ ppt	120
6	Final isoelectric ppt (growth hormone)	12

TABLE II

Biological Potency of Growth Hormone in Hypophysectomized Rats

Daily dose	No of rats	Average growth in 10 days
mg		*gm*
0 10	36	19 2
0 05	21	18 3
0 02	11	11 0
0 01	18	10 0

TABLE III

Assay of Growth Hormone on Proximal Epiphyseal Cartilage of Tibia

Daily dose	No of rats	Width of uncalcified cartilage: Mean	Width of uncalcified cartilage: Difference from control
mg		*μ*	*μ*
0 05	6	344	189
0 03	8	296	141
0 01	8	220	65
0 00	7	155	0

The growth activity of a typical preparation, as estimated by the body weight increments produced in hypophysectomized female rats, is given in Table II. It may be noted that a 0.010 mg. daily dose (nine injections) causes a 10 gm. increase in body weight in 10 days. The biological activity of the growth hormone preparation is also standardized by its action on the epiphyseal cartilage of hypophysectomized female rats (11). The results are summarized in Table III, it is evident that a 0.010 mg. daily dose for

4 days is sufficient to increase the width of the uncalcified portion of the proximal epiphyseal cartilage of the tibia 50 per cent over that of the control.

Homogeneity Studies

Electrophoresis—Electrophoretic studies were conducted in a Tiselius (12) apparatus with the scanning method of Longsworth (13). The fraction obtained from each step in the purification was examined carefully as to its electrophoretic homogeneity. The increase of growth potency, the removal of biologically active contaminants, and the decrease in the number of boundaries in electrophoresis were correlated so as to evaluate the usefulness of each purification step. In this way, one can ascertain the component in the electrophoresis patterns which represents the growth hormone. For instance, the material secured from step (5) possessed one

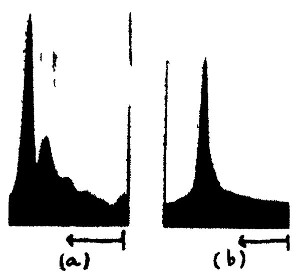


FIG. 1. Electrophoresis patterns of (*a*) a growth hormone fraction before isoelectric precipitation and (*b*) the same fraction after the isoelectric precipitation in acetate buffer of pH 4.0 and an ionic strength of 0.10, potential gradient about 6 volts per cm.; 120 minutes electrolysis.

main component together with two other components in smaller concentration, when subjected to electrophoretic examination in an acetate buffer of pH 4.0 and 0.10 ionic strength (see Fig. 1, *a*). After isoelectric precipitation of this same fraction, the final product retained only the main component without loss of growth potency (see Fig. 1, *b*).

The electrophoretic examination of the final product reveals no indication of the presence of other components. Fig. 2 gives some typical electrophoretic patterns of the ascending boundary when the electrophoresis proceeds in acetate buffer of pH 4.95 and an ionic strength of 0.10 at 1.5° for 540 minutes, while Fig. 3 is obtained in barbiturate buffer of pH 9.60 and an ionic strength of 0.10 after the current is on for 360 minutes.

When a 1 per cent growth hormone solution was electrolyzed in acetate buffer of pH 4.0 at a potential gradient of about 6 volts per cm. for 120 minutes, separate recovery was made of the solution in the anode limb, in

the cathode limb, and in the middle portion of the electrophoresis cell. After the nitrogen content was determined by micro-Kjeldahl analysis,

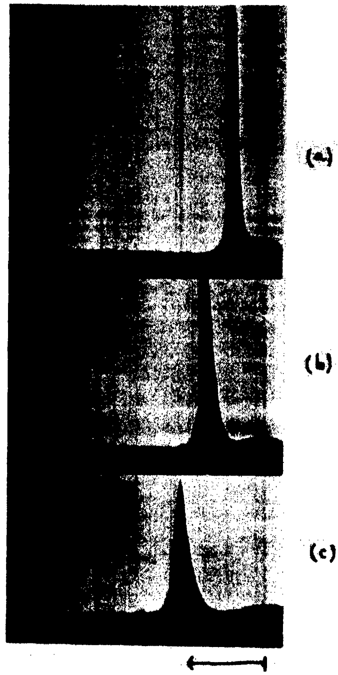


Fig. 2. Electrophoresis patterns of the ascending boundary of a growth hormone preparation in acetate buffer of pH 4.95 and an ionic strength of 0.10. Electrolysis for (*a*) 180 minutes, (*b*) 240 minutes, and (*c*) 540 minutes at a potential gradient of 2.5 volts per cm.

Fig. 3. Electrophoresis patterns of the ascending boundary of a growth hormone preparation in barbiturate buffer of pH 9.60 and an ionic strength of 0.10 for 360 minutes at a potential gradient of 2.6 volts per cm.

the fractions were assayed in hypophysectomized female rats for growth activity. It will be observed in Table IV that there was no significant

difference in the growth potency of these fractions. If the protein were not the hormone, a marked decrease or increase of growth activity should be obtained in one of the fractions.

Diffusion—The value of the sintered glass disk technique to indicate homogeneity of biologically active proteins has been emphasized by Northrop and Anson (14) and others. A 0.5 per cent growth hormone solution (acetate buffer of pH 4.0) was diffused through a sintered glass disk into 25 cc. of the same buffer which was replaced with fresh solution at definite time intervals. The amount of nitrogen diffused in each period was determined with the micro-Kjeldahl method. As shown in Table V, the amount of diffused nitrogen per day was practically constant indicating uniformity

TABLE IV

Assay of Protein Solutions in Different Sections of an Electrophoresis Cell

Daily dose, 0.05 mg.

Fraction	No. of rats	Average growth in 10 days
		gm.
Anode	4	16
Cathode	7	16
Center	8	18

TABLE V

Amount of Nitrogen Diffused in Successive Days with Growth Preparation

Sample	Diffusion period	Amount of N
	days	*mg.*
A	4	0.454
B	1	0.105
C	1	0.090
D	1	0.105

in the molecular size of the protein. There was also no appreciable difference in the growth potency of the protein solutions (0.05 mg. daily) before and after the diffusion experiment, as shown by an average growth in 10 days of 16 gm. for nine rats before diffusion and of 15 gm. for eight rats after diffusion. If the growth hormone molecule were actually smaller in size than the contaminating proteins, it would have diffused away and the remaining protein solution would have shown loss of growth-promoting activity.

Solubility The purity of the growth hormone preparation was also examined by the solubility method of Northrop (15). Three solvents were used, distilled water of pH 7.1, 3.8 M NaCl in 0.07 M phosphate buffer of

pH 6.4, and 4.8 M NaCl in 0.07 M phosphate buffer of pH 5.7. All measurements were made at 5°. The soluble nitrogen was analyzed by the micro-

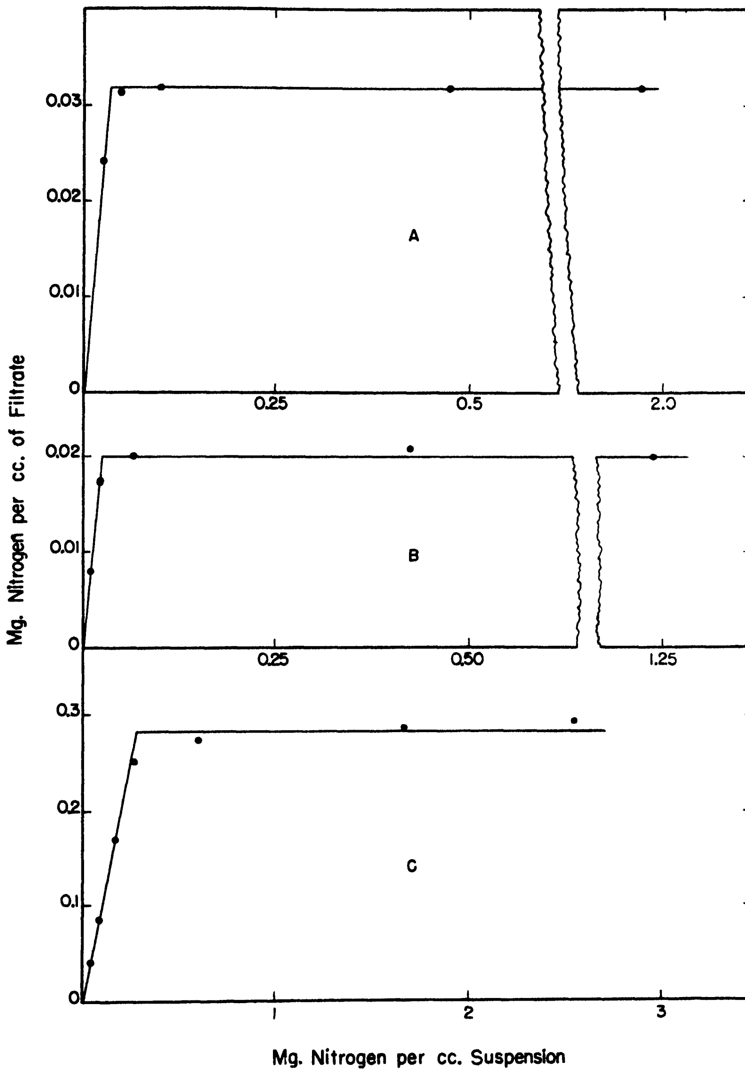


FIG. 4. Solubility curves of the growth hormone in various solvents at 5°. (*A*) solvent, distilled water, pH 7.1; (*B*) solvent, 4.8 M NaCl in 0.07 M phosphate buffer, pH 5.7; (*C*) solvent, 3.8 M NaCl in 0.07 M phosphate buffer, pH 6.4.

Kjeldahl method. The results presented in Fig. 4 indicate that the solubility of the growth hormone preparation is constant after the appearance

of the solid phase. Bioassays of the soluble and insoluble protein revealed no difference in the growth-promoting activity.

Particles of Growth Hormone

A 1.0 per cent growth hormone solution, at approximately pH 10, was immersed in a water bath of about 30°; 1 or 2 drops of 1 M HCl were added to bring the pH down to about 7.0; the precipitate was centrifuged off in a warm environment. The opalescent supernatant was set aside in a cold room (2–3°). After 2 or 3 days, the particles which had settled were found to be remarkably uniform in size and shape. These ball-shaped particles were assayed in hypophysectomized female rats and found to possess the

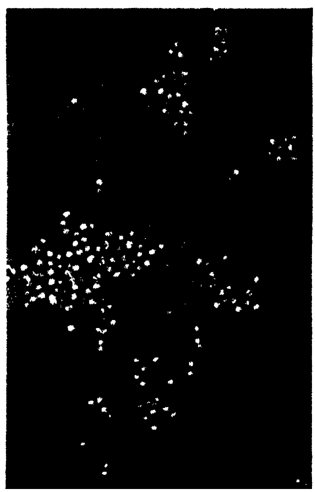


FIG. 5. The growth hormone particles ×150

same growth potency as the original solution. Fig. 5 shows a photomicrograph of the growth hormone particles.

Isoelectric Point and Molecular Weight

In a series of buffer solutions of different pH we have obtained the mobility of the growth hormone in a Tiselius electrophoresis apparatus. The ionic strength of the buffer was 0.10 and the temperature 1.5°. From Fig. 6, the isoelectric point of the growth hormone is estimated to be pH 6.85.

At the isoelectric point and in the presence of $(NH_4)_2SO_4$, the growth hormone is sufficiently soluble for osmotic pressure measurements. Under these conditions the molecular weight of the hormone was found to be 44,250, as calculated by the modified van't Hoff equation (16).

$$M = CdRT/100P$$

in which C is the number of gm. of solute dissolved in 100 gm. of solvent, M the molecular weight of the solute, d the density of the solvent, P the observed pressure in cm. of water, R and T having their usual significance. The results are summarized in Table VI; the value 44,250 is an average figure from four determinations with four different growth hormone prep-

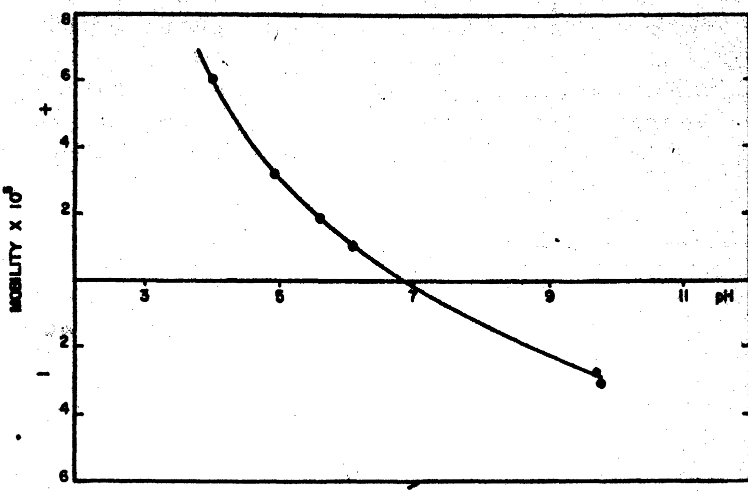


FIG. 6. The electrophoresis mobility of growth hormone as a function of pH at 1.5°.

TABLE VI

Osmotic Pressure of Growth Hormone Solutions at 2°

pH	Concentration of $(NH_4)_2SO_4$	Protein per 100 gm. solvent (C)	Pressures observed (P)	$C:P$	Mol. wt. calculated
	M	*gm.*	*cm.* H_2O		
6.64	0.40	0.485	2.70	0.180	43,500
6.80	0.40	0.845	4.64	0.182	44,000
7.00	0.40	0.550	2.99	0.184	44,500
6.65	0.80	0.350	1.95	0.180	45,000
Average..........					44,250

arations. The method of measuring the osmotic pressure was that employed in a previous investigation (17).

Some Analytical Data

The results are summarized in Table VII.

Carbon, hydrogen, sulfur, and nitrogen (Dumas) were obtained by dupli-

cate analyses.[1] The Kjeldahl nitrogen was an average of many determinations; selenium oxychloride in H_2SO_4 was used as the digestion mixture.

Amino N was obtained by the usual method in the Van Slyke apparatus. The value is an average of two determinations. The estimate of amide N was kindly carried out by Dr. H. S. Olcott in duplicate determinations. The number of acid and basic groups was kindly determined for us by Dr. H. Fraenkel-Conrat with a micro technique employing a dye titration method.

Tyrosine and tryptophane contents were averages of six determinations with two growth hormone preparations by Lugg's modification (18) of the method of Folin and Ciocalteu. Approximately 70 mg. of protein were

TABLE VII

Some Analytical Data on Growth Hormone

Constituent	Content
	per cent
C	46.35
H	7.07
S	1.30
N, Dumas	15.65
" Kjeldahl	15.50
Amino N	0.76
Amide "	1.20
No. of acid groups per 10,000 gm. protein	9.80
" " base " " 10,000 " "	13.40
Tyrosine	4.30
Tryptophane	0.92
Cysteine	0.00
Glutamic acid	13.40

used in each experiment and the color developed was read in a Cenco photelometer. The technique has been described in a previous paper (19).

When growth hormone was dissolved in ammoniacal solution in the presence of nitroprusside, no violet color was observed. Moreover, the growth hormone did not take up iodine in acid solution, indicating the absence of SH groups. It may therefore be concluded that the hormone contains no cysteine.

The glutamic acid was determined by Lewis and Olcott using a microbiological method (20). The value was derived from duplicate determinations.

[1] Analyzed by Dr. Carl Tiedcke of New York.

Effect of Trypsin and Pepsin

The solvent used for the pepsin experiments was acetate buffer of pH 4.0 and that for the trypsin, borate buffer (pH 8.5). The hormonal concentration was 0.9 mg. per cc., while the enzyme concentration was 0.4 mg. per cc. Both the trypsin and pepsin employed were commercial preparations, pepsin (Lilly) and trypsin (Pfanstiehl). The temperature of the incubation was 37° ± 1°. At the end of 90 minutes, solutions were neutralized and kept at 0° for assay. As shown in Table VIII, the growth potency as assayed in hypophysectomized female rats was greatly reduced after enzymatic treatment. These results are to be expected if the protein is the hormone.

Effect of Heat

It is generally believed that growth hormone is a thermolabile substance. The destructive effect of alkali has been noted by Evans, Meyer, and Simpson (21) who found that growth activity is greatly decreased when

TABLE VIII

Effect of Trypsin and Pepsin on Growth Hormone

Treatment	Daily dose	No. of rats	Average growth in 10 days
	mg.		*gm.*
Untreated	0.05	8	17
Pepsin	0.20	8	6
Trypsin	0.20	10	1

extracts are warmed to 37–50° at pH 11 or 12. On the other hand, Shipley (22) showed that growth potency is only reduced 50 per cent when extracts are boiled at pH 2.0 for 10 minutes. It appeared of interest to investigate the heat stability of the growth hormone in some detail.

The protein concentration used in boiling experiments was 1 mg. per cc. Three buffers were used: acetate buffer (pH 4.0) of ionic strength 0.10, phosphate buffer (pH 7.5) of ionic strength 0.10, and 0.1 M Na_2HPO_4 solution (pH 8.9). The hormone solutions were placed in 50 cc. test-tubes and immersed in boiling water for 10 minutes. After the solutions were cooled and neutralized, they were assayed in hypophysectomized female rats for growth potency. It was noted that all solutions became very cloudy during heating. The results summarized in Table IX indicate that the growth activity is practically lost under these conditions.

The protein was also subjected to heat treatment at lower temperature. The protein concentration was 0.2 mg. per cc. in phosphate buffer of pH 7.0 and an ionic strength of 0.10. The length of heat treatment was 60

minutes. It was observed that the solution remained perfectly clear up to 60°; turbidity began at 70° and flocculent precipitation occurred at 80°. On the other hand, the growth potency was not significantly reduced at 60° but was destroyed at 70° and 80° as judged by assay in hypophysectomized rats (see Table IX). It is of great interest to note that decrease in growth activity always coincided with the appearance of protein precipitation.

Finally, we determined the stability of growth hormone in acid or alkali solutions at 60°. Growth hormone (10 mg.) was dissolved in 50 cc. of 0.1 M NaCl with the aid of 1 drop of 1.0 M NaOH or 1.0 M HCl; clear solutions were immersed in water at 60° for 60 minutes. The final pH was determined and found to be 10.5 and 3.1 respectively. Solutions were neutralized and assayed for growth potency. As shown in Table IX, there

TABLE IX

Bioassay of Growth Hormone in Solutions of Different pH after Heating in Water Bath at Different Temperatures

Daily dose, 0.10 mg.

pH	Temperature	Appearance after heating	No. of rats	Average growth in 10 days
	°C.			gm.
7.5	100	Flocculent ppt.	10	−2
4.0	100	" "	12	4
8.9	100	" "	5	2
7.0	37	Clear	8	17
7.0	60	"	15	15
7.0	70	Cloudy	9	4
7.0	80	Flocculent ppt.	13	0
3.1	60	Clear	8	4
10.5	60	"	14	17

appeared to be no loss of activity at pH 10.5 but a great reduction of activity at pH 3.1.

Effect of Urea

Urea has long been considered a good denaturing agent, capable of destroying the biological activity (23) and decreasing the size of protein molecules (16, 24). On the other hand, urea has no effect on the biological activity of lactogenic hormone (25) or upon pepsin (26).

Growth hormone (5 mg.) was dissolved in 0.5 cc. of 6.66 M urea-phosphate buffer (pH 7.0) and the solution was allowed to stand at 22° for 24 hours. The solution was then diluted and assayed without the removal of urea. The results with 0.05 mg. daily indicate that the hormone appears to retain its growth potency after urea treatment (an average growth of 19 gm. was

obtained on five rats for the untreated preparation and of 17 gm. on six rats for the urea-treated preparation during a 10 day period). This conclusion confirms the findings of Fraenkel-Conrat *et al.* (9) with a less purified growth preparation.

SUMMARY

A method has been described for the isolation of a protein which caused the resumption of body growth and increase in the epiphyseal cartilage cells of the tibia in hypophysectomized rats. The protein is thus identified as the growth hormone. From osmotic pressure determinations the molecular weight is found to be 44,250. Electrophoresis experiments show an isoelectric point at pH 6.85. Elementary analysis and the content of tyrosine, tryptophane, and glutamic acid have been determined. The hormonal activity is destroyed by pepsin and trypsin. It is unstable at the temperature of boiling water. In buffer of pH 7, the protein hormone is coagulated at 70–80° and the growth potency thus destroyed. It is further found that the hormone is more stable in alkali than in acid medium. Like pepsin and lactogenic hormone, growth hormone retains its biological activity in urea solutions.

BIBLIOGRAPHY

1. Bates, R. W., Laanes, T., and Riddle, O., *Proc. Soc. Exp. Biol. and Med.*, **33**, 446 (1935).
2. Schooley, J. P., Riddle, O., and Bates, R. W., *Anat. Rec.*, **22**, suppl., 90 (1938).
3. Evans, H. M., *Proc. Assn. Res. Nerve and Ment. Dis.*, **17**, 175 (1936).
4. Li, C. H., and Evans, H. M., *Science*, **99**, 183 (1944).
5. Evans, H. M., Uyei, N., Bartz, Q. R., and Simpson, M. E., *Endocrinology*, **22**, 483 (1938).
6. Marx, W., Simpson, M. E., and Evans, H. M., *Endocrinology*, **30**, 1 (1940).
7. Evans, H. M., and Simpson, M. E., *Anat. Rec.*, **29**, 356 (1925).
8. Evans, H. M., Cornish, R. E., and Simpson, M. E., *Proc. Soc. Exp. Biol. and Med.*, **27**, 101 (1929).
9. Fraenkel-Conrat, H., Meamber, D. L., Simpson, M. E., and Evans, H. M., *Endocrinology*, **27**, 605 (1940).
10. Fevold, H. L., Lee, M., Hisaw, F. L., and Cohn, E. J., *Endocrinology*, **26**, 999 (1940).
11. Evans, H. M., Simpson, M. E., Marx, W., and Kibrick, E., *Endocrinology*, **32**, 13 (1943).
12. Tiselius, A., *Tr. Faraday Soc.*, **33**, 524 (1937).
13. Longsworth, L. G., *J. Am. Chem. Soc.*, **61**, 529 (1929).
14. Northrop, J. H., and Anson, M. L., *J. Gen. Physiol.*, **12**, 543 (1928–29).
15. Northrop, J. H., *J. Gen. Physiol.*, **13**, 739 (1930).
16. Burk, N. F., and Greenberg, D. M., *J. Biol. Chem.*, **87**, 197 (1930).
17. Li, C. H., Lyons, W. R., and Evans, H. M., *J. Biol. Chem.*, **140**, 43 (1941).
18. Lugg, J. W. H., *Biochem. J.*, **32**, 775 (1938).
19. Li, C. H., Lyons, W. R., and Evans, H. M., *J. Biol. Chem.*, **136**, 708 (1940).
20. Lewis, J. C., and Olcott, H. S., *J. Biol. Chem.*, **157**, 265 (1945).

21. Evans, H. M., Meyer, K., and Simpson, M. E., *Mem. Univ. California*, **11**, 59 (1933).
22. Shipley, R. A., *Endocrinology*, **31**, 629 (1942).
23. Lauffer, M. A., and Stanley, W. M., *Science*, **89**, 2311 (1939).
24. Wu, H., and Yang, E. F., *Chinese J. Physiol.*, **6**, 51 (1932).
25. Li, C. H., Lyons, W. R., and Evans, H. M., *J. Biol. Chem.*, **139**, 43 (1941).
26. Steinhardt, J., *J. Biol. Chem.*, **123**, 543 (1938).

THE EFFECT OF PYRIDOXINE DEFICIENCY ON TRANSAMINATION IN STREPTOCOCCUS LACTIS

By PHILIP P. COHEN and HERMAN C. LICHSTEIN

(*From the Laboratories of Physiological Chemistry and Bacteriology, Medical School, University of Wisconsin, Madison*)

(Received for publication, April 17, 1945)

Schlenk and Snell (1) have recently reported that tissues from pyridoxine-deficient rats show a decreased transaminase activity. While Braunstein and coworkers (2) have long maintained that a cofactor was required for transamination, Cohen (3) and more recently D. E. Green (personal communication) have been unable to demonstrate the presence of such a factor in purified preparations.

In view of the recent finding by Lichstein and Cohen (4) that bacteria are active in transamination, it seemed desirable to put to test the suggestion of Schlenk and Snell by determining the transaminase activity of pyridoxine-deficient bacteria. It was felt that this approach would not be complicated by the well known effects of vitamin deficiencies in animals, such as inanition and changes in fat, carbohydrate, protein, and water ratios in tissues.

Bellamy and Gunsalus (5) have shown that cell suspensions of *Streptococcus faecalis* R, harvested from a medium deficient in pyridoxine, decarboxylate tyrosine at a markedly reduced rate. Thus, it is possible to cultivate this organism in a medium of known pyridoxine level and to check the effect of this vitamin by determining the rate of tyrosine decarboxylation by the cells.

Because of these facts, a strain of *Streptococcus faecalis* R was chosen for study. By expressing enzyme activity in terms of bacterial nitrogen rather than weight, it was felt that the objections raised above to studies of vitamin-deficient animals would be ruled out.

Methods

Culture and Media—*Streptococcus lactis* R (American Type Culture Collection, No. 8043) was used in all experiments, since it has been identified as a strain of *Streptococcus faecalis* (6). The medium employed was the one suggested by Bellamy and Gunsalus (5), except that hydrolyzed casein (Bacto-casamino acids) was used in place of hydrolyzed gelatin. Although Snell and Guirard (7) have shown that this organism will grow in the absence of pyridoxine provided sufficient alanine is present, small amounts of the vitamin were added to the deficient media in order to insure adequate growth.

The organisms were acclimated to the various media by eight serial transfers before the experiments were carried out. In Experiment 1 the synthetic media employed contained respectively 4 and 40 γ of pyridoxine per 10 ml. In Experiment 2 three media were used, two synthetic containing 0.4 and 100 γ of pyridoxine per 10 ml. respectively, and a control medium composed of nutrient broth plus 0.5 per cent glucose and 1.0 per cent yeast extract (Bacto).

Preparation of Bacterial Suspensions—Large volumes of media (1 to 5 liters) were inoculated with *Streptococcus lactis* R, incubated for 24 hours at 37°, and the cells harvested by centrifugation in a Sharples ultracentrifuge at 4°. The recovered cells were washed twice with physiological saline solution, suspensions of suitable strength prepared, and nitrogen determinations made on aliquots by the micro-Kjeldahl procedure. The bacterial suspensions were prepared in equal volumes of physiological saline solution and 0.075 M phthalate buffer (pH 5.0) for the tyrosine decarboxylation studies. For the transamination experiments the cell suspensions were prepared in 0.1 M phosphate buffer, pH 7.4 for Experiment 1, and pH 8.0 for Experiment 2.

Tyrosine Decarboxylation—This was followed manometrically by determining the rate of CO_2 evolution (5). The main compartment of the Warburg cups contained 0.5 ml. of a M/30 suspension of tyrosine, 1 ml. of 0.075 M phthalate buffer (pH 5.0), and water to make 2.5 ml. The cell suspension (0.8 ml.) was placed in the side arm along with 0.2 ml. of buffer. Suitable controls without tyrosine were made in all instances. The gas phase was N_2, the temperature of incubation 38°, and readings were taken every 5 minutes for 50 minutes. All determinations were made at least in duplicate.

Transamination—The transamination reaction,

$$l(+)\text{-Glutamic acid} + \text{oxalacetic acid} \rightarrow l(-)\text{-aspartic acid} + \alpha\text{-ketoglutaric acid}$$

was studied by employing the methods described by Lichstein and Cohen (4) for bacteria.

Incubations were made in Warburg cups at 38° for 5 minutes with N_2 as the gas phase. Each cup contained 2 ml. of cell suspension in the main compartment. The substrates were placed in the side arm, 0.5 ml. of 0.12 M glutamic acid and 0.3 ml. of 0.2 M oxalacetic acid. Suitable controls without oxalacetic acid were made, and in most instances the experimental cups were run in triplicate. Aspartic acid formation was determined on deproteinized, boiled aliquots by using the chloramine-T method (3).

Results

The results of the tyrosine decarboxylation studies are presented in Table I. The difference in rate of activity between cells harvested from

pyridoxine-low and pyridoxine-high media was not as marked in Experiment 1 as in Experiment 2. Also, the Q_{CO_2} (N) values in all instances were higher than those reported by Gunsalus and Bellamy (6). There is no

TABLE I

Influence of Pyridoxine Level in Growth Medium on Tyrosine Decarboxylation by Streptococcus lactis R (American Type Culture Collection, No. 8043)

Experiment No.	Pyridoxine level of medium	Bacterial N per cup	Incubation time	Activity of cells	
				CO_2 evolved	Q_{CO_2} (N)
	γ per 10 ml.	mg.	min.	microliters	
1	4	0.43	10	93	1297
			15	133	1236
			30	217	1009
	40	0.39	10	187	2870
			15	262	2690
			30	375	1922
2	0.4	0.40	10	52	780
			15	70	700
	100	0.40	10	256	3840
			15	314	3140
	Control (glucose broth + yeast extract)	0.40	10	164	2460
			15	231	2310

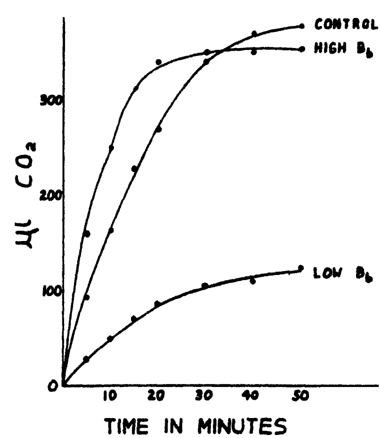


FIG. 1. Effect of pyridoxine level in growth medium on tyrosine decarboxylation by *Streptococcus lactis* R. (For the details refer to the text.)

doubt, however, that the cells from the pyridoxine-low media, in both instances, were actually deficient in their ability to decarboxylate tyrosine. This is perhaps more clearly seen in Fig. 1 which is a graphical presentation of the data obtained in Experiment 2.

The results of the transamination studies (Table II) clearly indicate no difference in activity between cells obtained from pyridoxine-low and pyridoxine-high media despite the very marked difference in their ability to decarboxylate tyrosine. The difference in the $Q_{\text{transamination (N)}}$ values for the two experiments is probably related to the pH of incubation (4).

DISCUSSION

The fact that pyridoxine-deficient *Streptococcus lactis* is capable of transaminating at the same rate as organisms grown on adequate media suggests that pyridoxine is not directly concerned in the transamination reaction. The simultaneous determination of tyrosine decarboxylation unequivocally establishes the organisms as deficient in pyridoxine.

TABLE II

Influence of Pyridoxine Level in Growth Medium on Transamination by Streptococcus lactis R (American Type Culture Collection, No. 8043)

Experiment No	Pyridoxine level of medium	Bacterial N per cup	CO_2 evolved	Activity of cells: Glutamic acid blank	Activity of cells: Aspartic acid formed	$Q_{\text{transamination (N)}}$
	γ per 10 ml	*mg*	*microliters*	*microliters CO_2*	*microliters CO_2*	
1	4	5.04	1507	1388	119	284
	40	7.5	1585	1388	197	315
2	0 4	3.41	1530	1330	200	704
	100	3.3	1517	1330	187	680
	Control (glucose broth + yeast extract)	3.2	1510	1330	180	675

Incubation time 5 minutes

While it is not our purpose to enter into a discussion of the validity of results obtained with tissues from vitamin-deficient animals, or with bacteria grown on vitamin-deficient media, it should be emphasized that tissues from vitamin-deficient animals often cannot be directly compared with those of control animals because of variation in water, fat, carbohydrate, and protein content of the deficient tissues. Since one is measuring enzyme activity of tissues, it would seem essential that activities be expressed in terms of nitrogen content rather than fresh weight. Further than this, however, one is still faced with the possibility that secondary effects only are being observed. Obviously, the surest basis for establishing the nature of a coenzyme is to purify the system and characterize it chemically. Thus, while Gunsalus *et al.* (8) have presented striking data in support of the idea that pyridoxine (specifically pyridoxal phos-

phate) is the coenzyme for tyrosine decarboxylase, recent work of Gale and Epps (9), who purified the system and split off the cofactor, have shown it to be free of phosphorus and to have properties which indicate that it is not pyridoxine.

It would thus appear from the present study that if pyridoxine plays any direct rôle in the transamination reaction this remains to be established. The unequivocal demonstration of such a rôle will have to await high purification of the transamination enzyme system.

SUMMARY

Data are presented which show that *Streptococcus lactis* R harvested from pyridoxine-low and that from pyridoxine-high media, while differing markedly in their ability to decarboxylate tyrosine, catalyzed the transamination reaction

$$l(+)\text{-Glutamic acid} + \text{oxalacetic acid} \rightarrow l(-)\text{-aspartic acid} + \alpha\text{-ketoglutaric acid}$$

at the same rate.

BIBLIOGRAPHY

1. Schlenk, F., and Snell, E. E., *J. Biol. Chem.*, **157**, 425 (1945).
2. Braunstein, A. E., *Enzymologia*, **7**, 25 (1939).
3. Cohen, P. P., *J. Biol. Chem.*, **136**, 565 (1940).
4. Lichstein, H. C., and Cohen, P. P., *J. Biol. Chem.*, **157**, 85 (1945).
5. Bellamy, W. D., and Gunsalus, I. C., *J. Bact.*, **48**, 191 (1944).
6. Gunsalus, I. C., and Bellamy, W. D., *J. Biol. Chem.*, **155**, 557 (1944).
7. Snell, E. E., and Guirard, B. M., *Proc. Nat. Acad. Sc.*, **29**, 66 (1943).
8. Gunsalus, I. C., Bellamy, W. D., and Umbreit, W. W., *J. Biol. Chem.*, **155**, 685 (1944).
9. Gale, E. F., and Epps, H. M. R., *Biochem. J.*, **38**, 250 (1944).

THE MICRO-KJELDAHL DETERMINATION OF THE NITROGEN CONTENT OF AMINO ACIDS AND PROTEINS*

By LILA MILLER AND JEAN ANNE HOUGHTON

(*From the Department of Biological Chemistry, Medical School, University of Michigan, Ann Arbor*)

(Received for publication, March 12, 1945)

The determination of the nitrogen content of proteins and amino acids is of fundamental importance for many investigations, inasmuch as the validity of other phases of the study often rests upon the accuracy of this determination. The Kjeldahl method for nitrogen has undergone many modifications since its inception, usually with the object of decreasing the period required for digestion. In the last 15 years, the wide-spread use of micro modifications of this method has introduced further variations in the procedure. The Kjeldahl method has had such universal acceptance that, in many publications, no details as to the accuracy of the particular procedure, the oxidizing and catalytic agents employed, or the time of heating are given.

Late in 1942, in connection with a problem in protein analysis, failure to obtain quantitative values for the nitrogen content of lysine dihydrochloride was encountered. In view of this and of the low results obtained for tyrosine also, especially in the presence of selenium as a catalyst (unpublished data), a careful study of the procedure as applied to these two amino acids was undertaken, a study which was extended subsequently to include other amino acids.

While this work was in progress, Van Slyke and coworkers[1] stated that neither lysine nor tryptophane gave quantitative values by the Kjeldahl analysis (1), the usual values being about 90 per cent of the theoretical, and that trial of practically all oxidizing and catalytic agents recommended in the literature for the Kjeldahl digestion did not, in their hands, improve the results. Chibnall *et al.* (2) in the following year discussed the conditions required for obtaining maximal, reproducible values of the nitrogen content of carefully purified proteins. The conditions reported here, which we have found satisfactory for a number of different amino acids, are not identical with those of Chibnall.

* This work was supported by a grant from the research funds of the Horace H. Rackham School of Graduate Studies, University of Michigan.

[1] The statement referred to is included in a foot-note on p. 142 (1).

EXPERIMENTAL

Reagents—

Sodium hydroxide. Solution A, 50 per cent sodium hydroxide (by weight). Solution B, 40 gm. of sodium hydroxide, 5 gm. of crystalline sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$), and 60 gm. of water.

Boric acid, 2 per cent solution. To each liter of solution, 11 ml. of the methyl red-bromocresol green mixture of Ma and Zuazaga (3) are added.[2]

Hydrochloric acid, 0.01 N.

Distillation—The first phase of this study was the determination of the limits of accuracy of the distillation procedure. The test material was pure ammonium sulfate dried at 105–110°. An all-glass apparatus was used.[3]

In preliminary studies a period of 4 minutes and 30 seconds for distillation was satisfactory. Later it was observed that, because of the fluctuations in the gas pressure, the heat from a single micro burner was so variable that in some cases the distillations were incomplete. To increase the conduction of heat to the water in the steam generator a coil of fine platinum wire 2¾ inches in length was sealed into the bottom of the steam generator. This wire was coiled inside of the flask and extended through to the outside about 1/16 of an inch. With more rapid generation of steam, special care had to be exercised in mixing thoroughly the mercury containing digests and the alkali (sodium hydroxide, Solution B) in order to be assured of complete neutralization of the acid before the temperature was greatly elevated. With attention to this point, the liberation of hydrogen sulfide from the acid digest was avoided.

For the apparatus equipped with the platinum coil, the following procedure was established. After the introduction of the solutions, the generator was first heated with one micro burner at full flame until the first trap was warm; then heat from a second micro burner was supplied. This second burner was adjusted to the point of just giving a two cone flame. The stop-watch was started when the first visible drop of condensate passed the center point of the bridge between the traps and the condenser. The distillation was allowed to proceed for 2 minutes, when the receiving flask was lowered so that the tip of the condenser was about 3 cm. above the acid. At the end of 2 minutes and 15 seconds the water was removed from the condenser, and, at the end of a period of 3 minutes, the receiving flask was lowered again so as to bring the tip of the delivery tube into the

[2] This indicator consists of 10 parts of 0.1 per cent solution of bromocresol green in alcohol and 2 parts of a similar solution of methyl red.

[3] The all-glass apparatus used in this study is the distilling head, M-3076, of the Scientific Glass Apparatus Company (Bloomfield, New Jersey), fitted with a 1 liter flask.

neck of the flask. This position was maintained for 30 seconds, giving a total elapsed time of 3 minutes and 30 seconds. The tip of the condenser was thoroughly washed with a fine stream of water when each of these changes in position was made.

All distillates, both blanks and experimental, were made up to the same volume, 40 to 50 ml., and titrated with 0.01 N hydrochloric acid.[4]

TABLE I

Recovery of Nitrogen from Standard Solutions of Ammonium Sulfate by Micro-Kjeldahl Method

1 ml. aliquots containing 0.5662 mg. of nitrogen were used in all cases.

	Nitrogen found	Recovery
	mg.	*per cent*
	0.5658	99.93
	0.5686	100.42
	0.5699	100.66
	0.5676	100.24
	0.5689	100.47
	0.5630	99.61
	0.5668	100.10
	0.5659	99.94
Average . .	0.5671	100.18

TABLE II

Reagents Used for Digestion in Micro-Kjeldahl Method

Reagent	Potassium sulfate	Crystalline cupric sulfate	Mercuric oxide	Sulfuric acid	Superoxol
	mg.	*mg.*	*mg.*	*ml.*	*drops*
A	100	100		1.0	3
B	200	200		1.0	3
C	110	110		1.0	3
D	300	100		1.0	3
E	500		50	1.5	

Representative data obtained from one solution under these conditions are presented in Table I. In this typical series the limits of accuracy appear to be -0.0037 to $+0.0032$ mg., with an average of $+0.0009$ mg., which is equivalent to 0.18 per cent above the theoretical.

These tests showed that the conditions of heating must be rigidly standardized and maintained, and that, with the established conditions for this

[4] The calibrated burette used is of 5 ml. capacity, graduated in hundredths.

particular piece of apparatus and source of heat, theoretical values for nitrogen of ammonium sulfate could be obtained.

Digestion—A study of the conditions for the digestion of samples was made with the quantities of salts, sulfuric acid, and superoxol given in Table II. The results with these combinations of reagents are shown in Table III. The inadequacies of cupric sulfate as the sole catalyst were evident in the analysis of lysine dihydrochloride and β-lactoglobulin, but not of alanine. It was not possible to obtain satisfactory analysis of lysine

TABLE III

Effect of Variation in Digestion Procedure on Nitrogen Values Obtained by Micro-Kjeldahl Method

The solutions analyzed contained 0.5 to 1.5 mg. of nitrogen per ml. For the reagents referred to by the letters A, B, C, D, and E, see Table II.

Substance	Reagents	No. of determinations	Total period of digestion	Nitrogen content			Recovery
				Minimal	Maximal	Average	
			hrs.	*per cent*	*per cent*	*per cent*	*per cent*
dl-Alanine	A	6	3.5-4.5	15.53	15.75	15.63	99.42
	E	12	2-8	15.55	15.83	15.70	99.87
	"	7	4-7	15.60	15.77	15.70	99.87
dl-Lysine dihydrochloride							
Solution I	B	2	4.5	10.34	10.36	10.35	80.92
	"	2	4.5	10.80	10.86	10.83	84.68
	E	2	4.5	12.51	12.53	12.52	97.89
" II	C	2	5	10.51	10.53	10.52	82.25
	A	2	7	9.92	11.09	10.50	82.10
	E	2	3.3	12.89	12.90	12.90	100.86
" III	D	2	5.8	10.08	10.13	10.11	79.05
	E	2	2.0	12.67	12.68	12.68	99.14
	"	2	5.8	12.67	12.71	12.69	99.22
β-Lactoglobulin							
Solution I	B	8	4-6	15.19	15.53	15.32	
	D	2	4	15.12	15.16	15.14	
	E	4	5.5	15.56	15.69	15.63	
" II	"	3	5.8-6.0	15.56	15.65	15.62	

dihydrochloride with cupric sulfate as catalyst. Similarly, the nitrogen values obtained for β-lactoglobulin with this catalyst were significantly lower than those obtained by the Dumas method (4).

In 1941, Clark (5) reported that, over a long period of time, he had successfully applied the Gunning-Arnold-Dyer modification of the macro-Kjeldahl method to the micro form of this method. On the basis of his report, digestion with 500 mg. of potassium sulfate, 50 mg. of mercuric oxide, and 1.5 ml. of sulfuric acid was tried. This combination of reagents is designated Reagent E in Tables II and III. The addition of 1 drop of

alcohol recommended by Clark (5) was made at the end of the 5th hour of digestion. In the presence of mercury, sodium hydroxide containing sodium thiosulfate (sodium hydroxide, Solution B) was necessary and, as already mentioned, the liberation of hydrogen sulfide must be avoided.

With alanine, the nitrogen values obtained with mercuric oxide as catalyst were but slightly higher than those obtained with cupric sulfate, whereas with mercuric oxide (Reagent E) the recovery of nitrogen of lysine dihydrochloride was quantitative. Likewise, with β-lactoglobulin, digestion with mercuric oxide gave higher nitrogen values.

To study the effect of the catalyst, two experiments were done in which the total periods of heating were the same and the catalyst was the only variable. From the results obtained for Solutions I and III of lysine dihydrochloride in Table III, the differences in the recoveries of nitrogen must be attributed to differences in catalysts and not to the different periods of heating. With β-lactoglobulin the large amount of charred material automatically necessitated a long period of digestion. The values for the β-lactoglobulin digested in the presence of mercury are in good agreement with the value of 15.63 per cent obtained by the Dumas method (4) and the recent value of 15.58 obtained by the Kjeldahl method (2) (Table V).

The period of digestion, a probable source of error, was studied with alanine, tryptophane, and tyrosine. With digestion periods of 2, 4, 5.5, and 8 hours, and Reagent E, the average recoveries of the nitrogen of alanine ranged between 99.53 and 100.57 per cent, with no correlation between length of digestion and recovery. On the other hand, with *l*-tryptophane and *l*-tyrosine, a correlation did exist. Total periods of heating of 2, 5, and 7 hours gave recoveries of 92.80, 97.96, and 97.67 per cent, respectively, for tryptophane, and 2.6, 4, 4.5, and 6.75 hours gave recoveries of 97.67, 98.06, 98.84, and 99.10 per cent, for tyrosine. On the basis of these results, a standard 6 hour period of digestion was chosen for subsequent work, counted from the time the charred material had cleared, or, if no charring occurred, 6 hours after the fumes of sulfur trioxide appeared. The total time in all cases would thus be more than 6 hours, with one exception longer than any of the digestions reported in Table III.

The procedure thus established was as follows. Solutions containing 0.4 to 1.4 mg. of nitrogen in aliquots of 1 or 2 ml. were digested with 500 mg. of potassium sulfate and 50 mg. of mercuric oxide and with 1.5 ml. of concentrated sulfuric acid (low nitrogen) with gentle heating until the water had evaporated and then with the full flame of the micro burners.[5]

[5] Digestion rack No. 7498, A. H. Thomas Company, has been found convenient as the heat from the micro burners is concentrated and not diffuse as in some types of racks tried. This particular rack has been used for all the experiments given in Tables IV and V.

At the end of 5 hours the flasks were cooled, 1 drop of ethyl alcohol was added, and heating continued 1 hour longer. At the end of digestion, the flasks were cooled, 5 ml. of distilled water added, and the flasks stoppered until distillation could be carried out. Usually the distillations were done on the following day. However, 3 days standing caused no detectable deviation in results.

Before the distillations were begun, the apparatus was steamed for at least 10 minutes and washed. With the tip of the delivery tube dipping into the boric acid solution (10 ml.), the digests were transferred to the apparatus with about 8 ml. of water divided into three portions. The funnel was then rinsed, and the solution of sodium hydroxide added. In the case of sodium hydroxide Solution A, 4 ml. were used, and of Solution B, 5 ml. The last traces of the alkali were washed into the reaction chamber and the total volume brought up to 32 ml., which, in this apparatus, prevented splashing into the trap and yet was adequate for washing all materials into the chamber.

The stop-cocks were closed, the heat applied, and the distillation was carried out as described above, followed by titration with 0.01 N hydrochloric acid.

Blanks identical with the experimental solutions with respect to solvent were carried through the whole procedure.

Application of Standardized Method

Amino Acids—This standardized procedure was applied to the analysis of representative amino acids and glutathione. Each compound was dried to constant weight either at 103–105° or in a vacuum desiccator over phosphorus pentoxide, as required by the nature of the particular compound. In each case, at least two different solutions of the compound were used to check sources of error from weighing. The data are presented in Table IV, in which each group of determinations represents the results with a single solution.

The alanine, isoleucine, leucine, lysine dihydrochloride, phenylalanine, and valine were synthetic preparations. The *l*-tyrosine, *l*-histidine hydrochloride monohydrate, cystine, and glutathione were prepared in this laboratory. In addition to the nitrogen values, the following criteria of purity were used for these respective compounds. The tyrosine was free from cystine; the sulfur content of cystine and of glutathione was 26.58 and 10.48 per cent, respectively; glutamic acid was free from chlorides. The *l*-tryptophane and *l*-glutamic acid were obtained from commercial sources. A preparation of *l*-arginine monohydrochloride was treated with norit, recrystallized in a manner similar to that recommended by Vickery (6) for the crystallization of free arginine, and dried before

TABLE IV

Nitrogen Content of Amino Acids and Glutathione As Determined by Standardized Micro-Kjeldahl Method

The solutions analyzed contained 0.4 to 1.0 mg. of nitrogen per ml. Unless otherwise indicated alcohol was added during the digestion as recommended by Clark (5).

Substance	No. of determinations	Nitrogen content			Recovery
		Minimal	Maximal	Average	
		per cent	*per cent*	*per cent*	*per cent*
dl-Alanine	2	15.73	15.77	15.75	100.13
	2	15.68	15.70	15.69	99.75
l-Arginine monohydrochloride	2	26.30	26.69	26.50	99.60
	2	26.27*	26.35*	26.31*	98.92*
	2	26.51	26.59	26.55	99.81
	2	26.52*	26.65*	26.59*	99.94*
	3	26.42	26.59	26.46	99.37
	3	26.33*	26.46*	26.36*	99.16*
Cystine	2	11.62	11.69	11.66	100.00
	2	11.56	11.63	11.60	99.49
l-Glutamic acid	2	9.56	9.57	9.57	100.49
	2	9.47	9.48	9.48	99.59
Glutathione	2	13.55	13.57	13.56	99.20
l-Histidine monohydrochloride monohydrate	2	20.05	20.11	20.08	100.15
	2	19.84	19.88	19.86	99.05
	2	20.03	20.13	20.08	100.15
dl-Isoleucine	4	10.72	10.77	10.73	100.47
	4	10.66	10.73	10.70	100.19
dl-Leucine	4	10.60	10.73	10.68	100.00
	1	10.71			100.27
dl-Lysine dihydrochloride	1	12.88			100.70
	2	12.82	12.82	12.82	100.23
dl-Phenylalanine	6	8.35	8.46	8.39	98.94
	2	8.39	8.45	8.42	99.29
l-Tryptophane	2	13.69	13.81	13.75	100.29
	2	13.77	13.83	13.80	100.58
	2	13.80	13.84	13.82	100.73
l-Tyrosine	6	7.64	7.77	7.70	99.61
	2	7.68	7.68	7.68	99.35
dl-Valine	4	11.88	11.92	11.90	99.49

* Same solution as that used for the determination in the line immediately preceding; no alcohol was used in digestion.

analysis. All of the compounds except valine and leucine gave negative tests for ammonia with Nessler's reagent. These two showed very slight traces of ammonia in the concentrations used for analysis.

The nitrogen values obtained for all of these compounds as shown in Table IV are considered quantitative. Three series of analyses of arginine

without the addition of alcohol recommended by Clark (5) are given in Table IV. The alcohol, when added at the end of the 5th hour of digestion, had no significant effect upon the results.

Proteins—Prior to the determination of nitrogen, the preparation of protein was spread in a thin layer and covered with a filter paper and allowed to equilibrate with the air for 48 to 72 hours. It was then stored in a closed bottle. For the moisture determination, these equilibrated samples were dried to constant weight at 103–105° in weighing bottles. For the determination of the ash content, the dried samples were transferred to crucibles (weighing by difference) and heated in a muffle oven at 550–600°.

TABLE V

Nitrogen Content of Proteins As Determined by Standardized Micro-Kjeldahl Method

All values are calculated on an ash- and moisture-free basis.

Protein	No. of determinations	Nitrogen content			Values reported in literature
		Minimal	Maximal	Average	
		per cent	*per cent*	*per cent*	*per cent*
β-Lactoglobulin	4	15.56	15.69	15.63	15.14–15.26 (8), 15.3 (9), 15.58 (2),
	3	15.56	15.65	15.61	15.60 (4)
Crystalline egg albumin	3	15.62	15.74	15.67	15.16 (10), 15.27 (11), 15.36-15.44
	4	15.63	15.67	15.65	(12), 15.4 (7), 15.60 (13), 15.51 (14), 15.64 (15), 15.71 (16), 15.76 (2)
Gliadin	4	17.66	17.86	17.79	17.58 (*), 17.66 (17), 17.87 (10)
	4	17.83	17.96	17.88	
Soy bean globulins (Illini)	6	16.85	17.00	16.96	16.93(†)
	5	16.89	17.14	17.00	
Glycinin	4	16.91	17.07	17.02	17.04 (10), 17.45 (17), 17.53 (18),
	4	16.97	17.22	17.08	16.60–17.74 (19)
Zein	4	15.93	15.99	15.97	16.13 (17)
	3	16.04	16.15	16.11	

* Osborne, T. B., private communication to H. B. Lewis.

† Vickery, H. B., and Pucher, G. W., private communication.

The samples for nitrogen analysis were taken at approximately the same time as those for the moisture and ash determinations to insure the validity of the corrections. Samples of 0.1 to 0.14 gm. of the protein were weighed into stoppered test-tubes, moistened with 1 ml. of water, mixed with 2 ml. of concentrated sulfuric acid, and hydrolyzed in a boiling water bath from 2.5 to 4 hours. The hydrolysates were transferred to 25 or 50 ml. volumetric flasks, diluted to volume, and 1 or 2 ml. aliquots used.

The results obtained by this procedure for β-lactoglobulin (prepared in this laboratory by one of the authors (M.)) are similar to those reported by Chibnall and others, 15.58 and 15.63 (2, 4) (Table V). The value for the

preparation of egg albumin is of special interest, as it is the same for which Calvery (7) previously reported 15.4 per cent nitrogen. As shown in Table V, our results for this protein are of the same order as those reported in some of the earlier papers as well as in certain recent ones, and are higher than a number of the other reported values.

Our value for the nitrogen of gliadin[6] is in good agreement with those of Jones and Moeller (10), but is higher than others reported in the literature. These differences may be due to the method of digestion or determination of moisture and ash, presumably the former. In the case of the soy bean globulins,[7] the slightly higher values we obtained in comparison with those of Vickery and Pucher do not, in our opinion, represent a significant difference. The agreement with the nitrogen for glycinin reported by Jones and Moeller is good (10). It is possible that the differences between our data for glycinin and others in the literature may be due to the procedure used or to differences in variety. Csonka and Jones (19) have claimed a difference in the nitrogen content of soy bean proteins, according to the variety of soy bean used as the source. The particular type of soy bean from which this preparation of glycinin[7] was obtained is not known. The nitrogen value for zein[7] is practically the same as that in the literature (17).

DISCUSSION

In micro-Kjeldahl analyses the conditions for distillation of the ammonia must be carefully checked with standard solutions of pure ammonium salts such as ammonium sulfate, and the established conditions must be rigidly maintained. The procedure for distillation recommended by this paper applies only to the particular piece of apparatus used here with our particular source of heat. Change to another piece of apparatus or to other conditions requires a revaluation of the entire procedure.

This study does not support the assumption that the catalyst and the period of digestion are necessarily identical for all compounds of the same general class. As reported here, the results for alanine are the same whether the catalyst is mercuric oxide or cupric sulfate, whereas a marked difference in nitrogen values occurs under the same conditions with lysine dihydrochloride and β-lactoglobulin. This observation is not new, but it has not been generally stressed. The necessity of the presence of mercury

[6] We are indebted through Professor H. B. Lewis to the late Dr. T. B. Osborne for the gliadin. The nitrogen value according to the analysis of Osborne was 17.58 on the moisture- and ash-free basis.

[7] We are indebted through Professor H. B. Lewis to Dr. H. B. Vickery for the soy bean globulins, which had been prepared in research carried out for the Food and Nutrition Board of the National Research Council, as well as for the glycinin and the zein.

in the analysis of certain proteins was pointed out by Pregl (20), who stated that cupric sulfate was entirely satisfactory in the cases of most of the other compounds studied. Weissman and Schoenheimer (21) have observed that with cupric sulfate and selenium long periods of digestion are required for lysine, but 2 hours are sufficient with mercuric sulfate.

The recommended period of heating is shorter than that used by Chibnall *et al.* (2). With their combination of potassium sulfate, sodium selenate, and sulfuric acid, 8 hours or longer were required for digestion. Although quantitative results for the nitrogen content of lysine dihydrochloride were obtained with a total period of heating of 2 hours, it cannot be assumed that this is sufficient to insure complete conversion of the nitrogen of all of the amino acids to ammonium sulfate, as one might be led to infer from Pregl's text (20). Tyrosine and tryptophane, for example, do yield quantitative nitrogen values with 6 hours digestion but not with shorter periods. It is also of interest that the time required for digestion of these amino acids is much longer than Clark (5) found necessary for the oximes, purines, pyrimidines, and other compounds he studied. It should be noted that he recommended 1 hour of heating, stating that 2 hours had no detrimental effect, and that he analyzed neither amino acids nor proteins.

SUMMARY

1. Conditions for the digestion and distillation in the micro-Kjeldahl method as applied to amino acids and proteins have been studied.

2. With mercuric oxide as the catalyst, quantitative values for the nitrogen of lysine dihydrochloride were obtained, but not with cupric sulfate. Likewise, higher values were obtained for the nitrogen content of β-lactoglobulin with the use of mercury as the catalyst.

3. With the standardized conditions employed here, quantitative values for the nitrogen content were obtained for alanine, arginine hydrochloride, cystine, glutamic acid, histidine hydrochloride monohydrate, isoleucine, leucine, lysine dihydrochloride, phenylalanine, tryptophane, tyrosine, valine, and glutathione.

4. The nitrogen values obtained by this procedure for the proteins β-lactoglobulin, egg albumin, gliadin, the soy bean globulins, and zein are compared with those reported in the literature.

BIBLIOGRAPHY

1. Van Slyke, D. D., Hiller, A., and Dillon, R. T., *J. Biol. Chem.*, **146**, 137 (1942).
2. Chibnall, A. C., Rees, M. W., and Williams, E. F., *Biochem. J.*, **37**, 354 (1943).
3. Ma, T. S., and Zuazaga, G., *Ind. and Eng. Chem., Anal. Ed.*, **14**, 280 (1942).
4. Brand, E., and Kassell, B., *J. Biol. Chem.*, **145**, 365 (1942).
5. Clark, E. P., *J. Assn. Official Agr. Chem.*, **24**, 641 (1941).
6. Vickery, H. B., and Leavenworth, C. S., *J. Biol. Chem.*, **76**, 701 (1928).

7. Calvery, H. O., Block, W. D., and Schock, E. D., *J. Biol. Chem.*, **113**, 21 (1936).
8. Pedersen, K. O., *Biochem. J.*, **30**, 961 (1936).
9. Palmer, A. H., *J. Biol. Chem.*, **104**, 359 (1934).
10. Jones, D. B., and Moeller, O., *J. Biol. Chem.*, **79**, 429 (1928).
11. Bernhart, F. W., *J. Biol. Chem.*, **132**, 189 (1940).
12. Hendrix, B. M., and Dennis, J., *J. Biol. Chem.*, **126**, 315 (1938).
13. Taylor, G. L., Adair, G. S., and Adair, M. E., *J. Hyg.*, **32**, 340 (1932).
14. Osborne, T. B., and Campbell, G. F., *J. Am. Chem. Soc.*, **22**, 422 (1900).
15. Sørensen, S. P. L., and Høyrup, M., *Compt.-rend. trav. Lab. Carlsberg*, **12**, 164 (1915–17).
16. Vickery, H. B., and Shore, A., *Biochem. J.*, **26**, 1101 (1932).
17. Osborne, T. B., The vegetable proteins, Monographs on biochemistry, London and New York, 2nd edition, 72 (1924).
18. Osborne, T. B., and Campbell, G. F., *J. Am. Chem. Soc.*, **20**, 419 (1898).
19. Csonka, F. A., and Jones, D. B., *J. Agr. Res.*, **46**, 51 (1933).
20. Pregl, F., Die quantitative organische Mikroanalyse, Berlin, 3rd edition, 121 (1930).
21. Weissman, N., and Schoenheimer, R., *J. Biol. Chem.*, **140**, 779 (1941).

THE INHIBITION OF THE GROWTH OF YEAST BY THIENYLALANINE

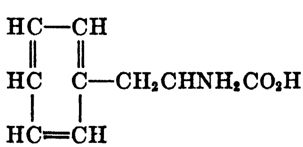
By VINCENT du VIGNEAUD, HERBERT McKENNIS, Jr., SOFIA SIMMONDS, KARL DITTMER, and GEORGE BOSWORTH BROWN

(*From the Department of Biochemistry, Cornell University Medical College, New York City*)

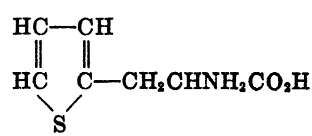
(Received for publication, April 16, 1945)

Within recent years it has been recognized that certain compounds related structurally to the vitamins or to other bacterial growth factors can inhibit the biological action of those essential substances.[1] One of the early experiments indicative of a metabolic antagonism between an essential amino acid and a related compound was reported by Dyer (4), who initiated biochemical studies on the relationship between methionine (S-methylhomocysteine) and ethionine (S-ethylhomocysteine). She observed that ethionine was toxic to young rats maintained on a methionine-deficient diet. This toxicity was offset by the addition of methionine to the experimental diet. Later, the "antimethionine" effect of ethionine was shown also for *Escherichia coli* by Harris and Kohn (5), who reported that the inhibition of bacterial growth by ethionine was completely overcome by methionine. More recently, it has been reported by Roblin *et al.* (6) that the oxygen analogue of methionine (α-amino-γ-methoxybutyric acid) also exerts an inhibitory effect on the growth of certain microorganisms which is reversed by methionine.

We wish to report here the results of similar studies concerned with another essential amino acid, phenylalanine, and its isostere, β-2-thienyl-



Phenylalanine



β-2-Thienylalanine

alanine.[2] At the time this work was begun, Barger and Easson (7) already had carried out preliminary investigations on the metabolism of thienylalanine in the rabbit. They obtained a persistent ninhydrin reaction in the urine following intravenous administration of the compound,

[1] This subject has been reviewed recently by Wagner-Jauregg (1), McIlwain (2), and Woolley (3).

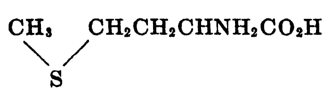
[2] This compound hereafter is designated as thienylalanine for brevity. The metabolism of the β-3-thienylalanine has not yet been investigated.

but were unable to isolate any thiophene derivative from it. 2-Thiophenic acid, on the other hand, was metabolized similarly to benzoic acid in that it gave rise to the thiophene analogue of hippuric acid.

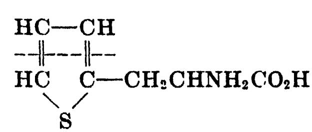
Our first experiments were designed to test the ability of thienylalanine to replace phenylalanine in the diet of the growing rat. It was found that the thienyl compound could not be used in lieu of the phenyl compound, since young rats lost weight rapidly when placed on an otherwise complete diet in which thienylalanine had been substituted for phenylalanine.

An attempt then was made to determine whether thienylalanine exerts an "antiphenylalanine" effect. Preliminary experiments with rats gave inconclusive results, although some indications of an antagonism were observed. A more extensive investigation of this point is necessary. However, a definite antagonistic effect on the part of thienylalanine was obtained with *Saccharomyces cerevisiae.* We noted inhibition of growth when thienylalanine was added to a medium which ordinarily permits good growth of yeast. The inhibitory action of thienylalanine was reversed by phenylalanine. Tyrosine had no effect on the toxicity of the thienyl compound. The effect of thienylalanine on the growth of other microorganisms is now being studied.

Yuan and Li (8), in 1937, pointed out that examination of the formula of thienylalanine reveals a structural similarity to the sulfur-amino acid, methionine.



Methionine



β-2-Thienylalanine

They suggested that thienylalanine might serve as a dietary source of methionine. The results of further biochemical studies by these workers apparently have not appeared in the literature, and we have, therefore, taken occasion to test such a possibility during the course of our investigations. Negative results were obtained. Young rats did not grow when placed on an otherwise complete diet in which thienylalanine was the only sulfur-containing amino acid.

In view of the numerous potentialities of thienylalanine as an inhibitor of pathogens and as a possible tool for the study of phenylalanine metabolism, both normal and abnormal, it seemed advisable to try to improve the existing methods for its preparation. Syntheses have been described by Yuan and Li (8) and by Barger and Easson (7). Both groups made use of the Erlenmeyer azlactone method (9), and, in addition, Barger and Easson adapted the hydantoin method of Wheeler and Hoffman (10).

Methods for the synthesis of 2-thiophenealdehyde were explored first, since this intermediate can be employed in either the azlactone or the hydantoin synthesis. In studying Barger's procedure (7) for the preparation of 2-thiophenealdehyde from 2-thienylglyoxylic acid, we found that increased yields could be obtained by decarboxylating the glyoxylic acid in aniline as described by Bouveault (11) for the preparation of aromatic aldehydes. It was found expedient, also, to prepare 2-thiophenealdehyde by means of the Grignard reagent from 2-iodothiophene as recommended by Yuan and Li (8). The yields from thiophene to aldehyde are somewhat lower by the latter procedure, but greater convenience is achieved.

Early in our attempts to carry out the condensation of 2-thiophenaldehyde with hydantoin in the presence of acetic anhydride and sodium acetate, as described by Barger and Easson (7), it became apparent that, in our hands, the procedure was unsatisfactory. Great variations in yields were encountered. Such difficulties in amino acid synthesis by the hydantoin method have been encountered previously (12), and it appeared probable that conditions for the preparation of 5-(2-thienylmethylene)hydantoin could be improved considerably.

Since 2-furfuraldehyde is the readily obtainable heterocyclic aldehyde most similar to 2-thiophenealdehyde, model experiments were carried out with the oxygen heterocycle. Deulofeu (13) reported the condensation of furfuraldehyde and hydantoin at 110° in the presence of sodium acetate alone. We were able to improve the yield of 5-(2-furfurylidene)hydantoin by carrying out the condensation at 140°. Under the same conditions we were able to effect the condensation of 2-thiophenealdehyde to give almost pure 5-(2-thienylmethylene)hydantoin in 75 per cent of the theoretical yield.

Barger and Easson reduced the thienylmethylenehydantoin with sodium amalgam, and obtained the desired amino acid by heating the resultant 5-(2-thienylmethyl)hydantoin with strong barium hydroxide solution. By means of the ammonium sulfide reduction which had been applied to the preparation of phenylalanine by Boyd and Robson (14), we obtained β-2-thienylalanine from the unsaturated hydantoin in one step. Our over-all yield from aldehyde to amino acid was 54 per cent of the theoretical amount. The over-all yield obtained by Barger and Easson is calculated to be 17 per cent of the theoretical amount. Yuan and Li obtained 15 per cent of the theoretical yield by the azlactone method (calculated from the acetal to the amino acid).

EXPERIMENTAL

Growth Studies; Feeding of Thienylalanine in Place of Phenylalanine—Six young rats were placed on a basal diet of the following percentage com-

position: amino acid mixture (as described by du Vigneaud, Chandler, Moyer, and Keppel (15) but containing no phenylalanine) 22.1, *dl*-methionine 0.6, *l*(−)-cystine 0.6, dextrin 25.7, sucrose 15.0, salt mixture (Osborne and Mendel (16)) 4.0, and corn oil (Mazola) 30.0. The fat-soluble vitamins A, D, E, and K were included in the diet as described previously (17), and the water-soluble vitamins of the B complex were supplied by two pills (18) daily, each of which contained 25 mg. of choline chloride in addition to 12.5 mg. of "modified ryzamin-B." After 4 days, during which time all animals but one lost weight, phenylalanine (1.5 per cent) was added

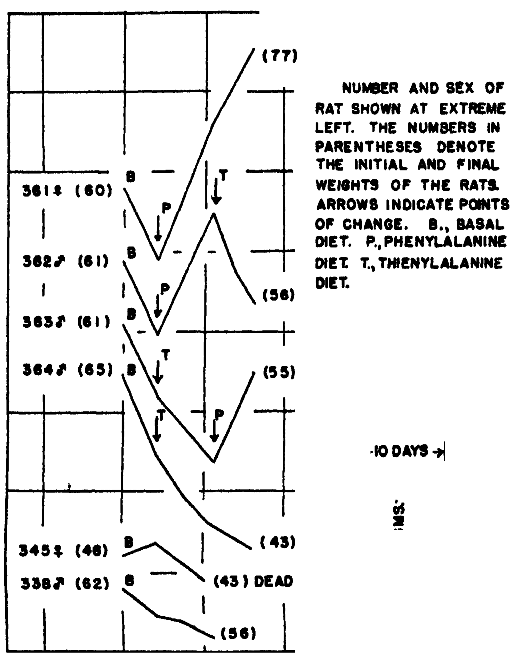


Fig. 1. Growth curves of rats fed thienylalanine in place of phenylalanine

to the diet of two animals, thienylalanine (1.5 per cent) was added to the diet of two animals, and two animals were continued on the basal diet. The control rats on the basal diet and those receiving thienylalanine continued to lose weight. Replacement of thienylalanine by phenylalanine resulted in gain of weight, while replacement of phenylalanine by its thiophene isostere resulted in loss of weight, as shown in Fig. 1. The daily food consumption of each animal is given in Table I. The urine of the rats receiving thienylalanine was collected, but no N-acetylthienylalanine could be isolated from it.

Feeding of Thienylalanine in Place of Methionine—Four young rats were given a diet similar to the basal diet described above, but with the following modifications: phenylalanine (1.5 per cent) was included in the diet of all four rats; methionine and cystine were replaced by thienylalanine (1.4 per cent) in the diet of two animals, while cystine was removed from the diet of the other two animals and the methionine content was raised to 1.4 per cent. The vitamin supplement was exactly the same as that used in the preceding experiment. The daily food consumption of these animals is given in

TABLE I

Food Consumption; Feeding of Thienylalanine in Place of Phenylalanine

Rat No. and sex	Days*	Supplement to basal diet	Average daily food consumption
			gm.
361 ♀	4–16	Phenylalanine	5.7
362 ♂	4–11	"	4.7
	11–16	Thienylalanine	3.4
363 ♂	4–11	"	3.1
	11–16	Phenylalanine	4.4
364 ♂	4–16	Thienylalanine	3.1
345 ♀	4–10		3.2
338 ♂	4–11		4.2

* The data for the first 4 days have been omitted, inasmuch as all animals were receiving the basal diet during this period.

TABLE II

Food Consumption; Feeding of Thienylalanine in Place of Methionine

Rat No. and sex	Supplement to basal diet	Average daily food consumption
		gm.
440 ♀	Methionine	4.2
441 ♀	"	4.3
442 ♂	Thienylalanine	2.7
443 ♂	"	2.1

Table II. The growth curves for this experiment, shown in Fig. 2, indicate that thienylalanine does not serve as a source of sulfur in place of methionine or of homocystine.

Experiment with Yeast—A 24 hour culture of *Saccharomyces cerevisiae* strain 139 was used for all tests. The yeast was grown in the media described by Snell, Eakin, and Williams (19), with one modification; namely, the aspartic acid content of the medium was doubled. After 16 hours incubation at 30°, yeast growth was measured turbidimetrically with a

Klett-Summerson photoelectric colorimeter. In Figs. 3 and 4 yeast growth is expressed in colorimeter units.

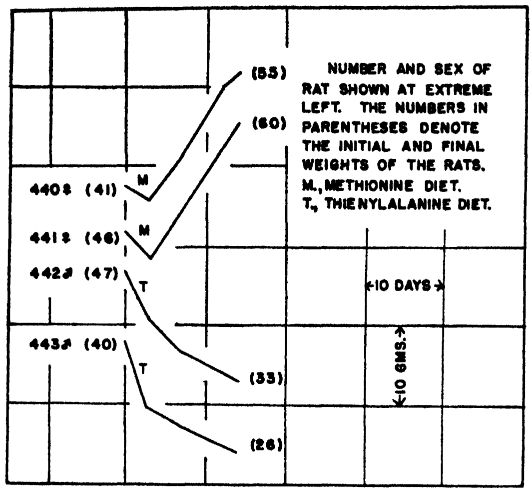


FIG. 2. Growth curves of rats fed thienylalanine in place of methionine

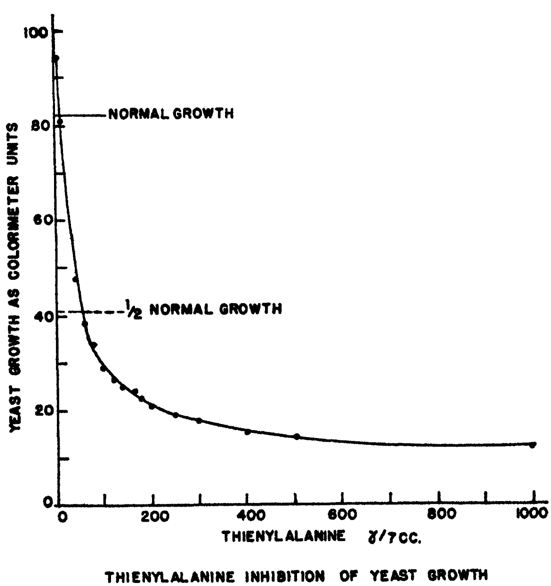
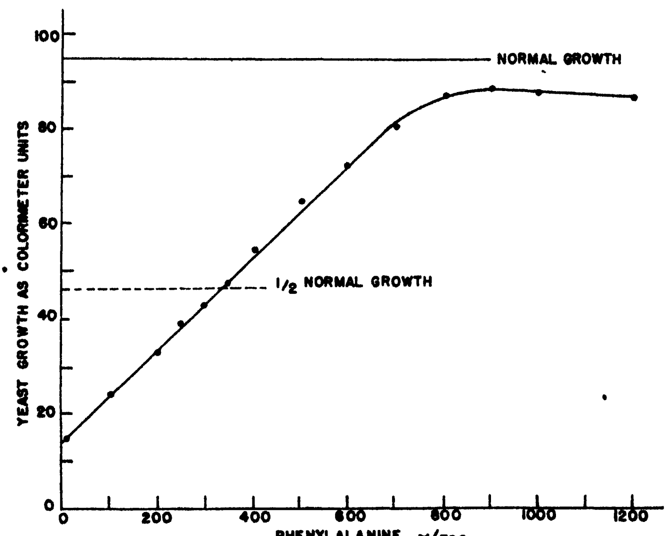


FIG. 3

The effect of increasing amounts of thienylalanine on the growth of yeast is shown in Fig. 3. It will be noted that 55 γ of thienylalanine per

7 cc. of medium reduced the growth to 50 per cent of normal. With amounts greater than 400 γ per 7 cc., no further inhibition was observed.

The addition of increasing amounts of phenylalanine to culture tubes which already contained 400 γ of thienylalanine per 7 cc. resulted in a gradual reversal of the inhibitory effect of thienylalanine (Fig. 4). The data represented in this figure indicate that approximately 800 γ of phenylalanine are required to reverse almost completely the toxicity of 400 γ of thienylalanine. The presence of large amounts of phenylalanine (0.8 to 1.2 mg. per 7 cc.) in the basal medium apparently caused a slight inhibition of growth, which may explain why the toxic effect of thienylalanine was



PHENYLALANINE REVERSAL OF YEAST GROWTH INHIBITION PRODUCED BY 400γ THIENYLALANINE PER 7 CC.

FIG. 4

never reversed completely by phenylalanine (see Fig. 4). *l*-Tyrosine in amounts as high as 800 γ per 7 cc. of medium had no effect on the toxic action of thienylalanine.

50 γ of thienylalanine remained inhibitory to yeast growth when the organism was grown in a medium which contained, in addition to the basal constituents, a mixture of amino acids having the following composition (the figures represent the amounts in micrograms per 7 cc.): *l*(−)-cystine 225, glycine 225, *l*(−)-hydroxyproline 225, *dl*-isoleucine 450, *dl*-methionine 450, *l*(−)-tyrosine 225, *dl*-alanine 450, *l*(+)-arginine monohydrochloride 225, *l*(+)-aspartic acid 900, *l*(+)-glutamic acid 2250,

$l(-)$-histidine monohydrochloride monohydrate 225, $l(-)$-leucine 225, $l(+)$-lysine monohydrochloride 225, $l(-)$-proline 225, *dl*-serine 450, *dl*-threonine 450, $l(-)$-tryptophane 225, and *dl*-valine 450. In the absence of thienylalanine, this mixture of amino acids stimulated the growth of yeast above the level obtained with the basal medium. The effect of the individual amino acids in concentrations much greater than that employed in the mixture is being investigated.

Preparation of 2-Thiophenealdehyde; (a) from 2-Acetothienone—2-Thienylglyoxylic acid, obtained by the oxidation of 52 gm. of 2-acetothienone (7), was dissolved in 500 cc. of aniline and heated at atmospheric pressure to distil all of the water formed. Most of the aniline then was distilled from the mixture under the vacuum of a good water pump at as low a temperature as possible. The residue was taken up in 500 cc. of 25 per cent H_2SO_4 and allowed to stand overnight. The acidic solution then was extracted repeatedly with ether, and the ether extract was evaporated to give 27.9 gm. of 2-thiophenealdehyde.

(*b*) *From 2-Iodothiophene*—The Grignard reagent from 121.1 gm. of 2-iodothiophene was treated with ethyl orthoformate according to the procedure of Yuan and Li (8). The yield of 2-thiophenealdehyde diethyl acetal, b.p. 90–92° at 9 mm., was 61.3 gm. This represents 57 per cent of the theoretical amount. Upon acid hydrolysis in the absence of oxygen, the acetal afforded 92 per cent of the theoretical amount of 2-thiophenealdehyde, b.p. 192–193°.

5-(2-Furfurylidene)hydantoin—An intimate mixture of 10.5 gm. of 2-furfuraldehyde, 10.5 gm. of hydantoin, and 10 gm. of anhydrous sodium acetate was heated at 140° for about 30 minutes. The mixture was allowed to cool and then was extracted with a total of 250 cc. of water. The residue, which melted at 231–237°, weighed 15.1 gm. This represents 78 per cent of the theoretical amount. Purification of the colored product was effected by recrystallization from alcohol. The colorless crystals melted at 239–240°.

$C_8H_6N_2O_3$	Calculated.	C 53.92,	H 3.40,	N 15.72
(178.2)	Found.	" 54.06,	" 3.67,	" 15.81

Deulofeu (13) reported that the compound melts at 230° after recrystallization from acetic acid and no analysis was given. Wheeler and Hoffman (10) obtained yellow prisms, m.p. 232°, the nitrogen content of which was shown to agree with the theoretical value.

5-(2-Thienylmethylene)hydantoin—To 18.4 gm. of 2-thiophenealdehyde (freshly distilled in an inert atmosphere) were added 15.8 gm. of finely powered hydantoin and 15.1 gm. of finely powdered sodium acetate. The intimate mixture, heated at 140–145° in an atmosphere of dry nitrogen for

70 minutes, first liquified and finally resolidified. The resultant magma was extracted with a total of 500 cc. of water and finally with 200 cc. of benzene. The residue consisted of almost colorless crystals of 5-(2-thienylmethylene)hydantoin, m.p. 253–255°. Admixture with a sample prepared according to Barger and Easson (7) caused no depression of the melting point. The yield was 23.7 gm., or 75 per cent of the theoretical amount.

β-2-Thienylalanine; (a) from 5-(2-Thienylmethyl)hydantoin—5.0 gm. of 5-(2-thienylmethyl)hydantoin prepared according to Barger and Easson (7) were dissolved in a mixture of 220 cc. of water and 25 cc. of concentrated ammonia water. The solution was cooled to 0°, was saturated with H_2S, and then was heated at 100° in a glass-lined autoclave for 5 days. The mixture next was filtered and concentrated *in vacuo* to a small volume. On addition of alcohol the amino acid precipitated. This material was dissolved in hot water, was filtered, and was reprecipitated by addition of alcohol. The yield of purified compound was 4.0 gm., representing 92 per cent of the theoretical amount.

$C_7H_9O_2NS$ (171.2) Calculated, S 18.7; found, S 18.9

(b). From 5-(2-Thienylmethylene)hydantoin and Ammonium Sulfide at 100°—7.7 gm. of 5-(2-thienylmethylene)hydantoin were dissolved in a mixture of 50 cc. of concentrated ammonia water and 75 cc. of water. The mixture was saturated with H_2S at 0°, and then was heated at 100° in a glass-lined autoclave for 64 hours. The mixture was filtered, and the filtrate was concentrated to dryness under diminished pressure. The hot aqueous extract of the residue was treated with decolorizing carbon, and then was concentrated to a small volume. β-2-Thienylalanine was precipitated by the addition of alcohol. The yield of colorless product was 3.4 gm., representing 50 per cent of the theoretical amount.

Calculated, S 18.7; found, S 18.3

(c) From 5-(2-Thienylmethylene)hydantoin and Ammonium Sulfide at 120°—In a sealed tube were placed 4.0 gm. of 5-(2-thienylmethylene)hydantoin and 80 cc. of ammonium sulfide solution (prepared by saturating 16 per cent ammonia water with H_2S at 0°). The mixture was heated at 120° for 88 hours and then was evaporated on the hot-plate until no more hydrogen sulfide was evolved. The residue was diluted with boiling water to a volume of approximately 100 cc. The hot solution was treated with decolorizing charcoal, was filtered, and was concentrated. Water was replaced by alcohol until the alcohol concentration was approximately 80 per cent. Upon cooling, the solution deposited colorless crystals, micro melting point 243–245°. The yield was 2.53 gm. This represents 72 per

cent of the theoretical amount. The mother liquors from several batches were combined and concentrated to dryness under diminished pressure. The gummy residue was recycled to yield additional quantities of the amino acid.

Calculated, S 18.7; found, S 18.7

SUMMARY

The addition of thienylalanine to a medium adequate for good growth of *Saccharomyces cerevisiae* caused marked inhibition of growth which was prevented by phenylalanine.

Thienylalanine, when added to the diet of the young rat, was incapable of supporting growth in lieu of either phenylalanine or methionine.

The synthesis of thienylalanine was reinvestigated and improved in regard to both yield and convenience.

The authors wish to express their appreciation to Dr. Julian R. Rachele and Mr. Roscoe C. Funk, Jr., for the microanalyses, and to acknowledge the valuable assistance of Mrs. Glenn Ellis and Miss Carol Tompkins in the microbiological phases of the work.

BIBLIOGRAPHY

1. Wagner-Jauregg, T., *Naturwissenschaften*, **31**, 335 (1943).
2. McIlwain, H., *Biol. Rev. Cambridge Phil. Soc.*, **19**, 135 (1944).
3. Woolley, D. W., *Science*, **100**, 579 (1944).
4. Dyer, H. M., *J. Biol. Chem.*, **124**, 519 (1938).
5. Harris, J. S., and Kohn, H. I., *J. Pharmacol. and Exp. Therap.*, **73**, 383 (1941).
6. Roblin, R. O., Jr., Lampen, J. O., English, J. P., Cole, Q. P., and Vaughan, J. R., Jr., *J. Am. Chem. Soc.*, **67**, 290 (1945).
7. Barger, G., and Easson, A. P. T., *J. Chem. Soc.*, 2100 (1938).
8. Yuan, H. C., and Li, H. C., *J. Chinese Chem. Soc.*, **5**, 214 (1937).
9. Erlenmeyer, E., Jr., *Ann. Chem.*, **275**, 1 (1893).
10. Wheeler, H. L., and Hoffman, C., *Am. Chem. J.*, **45**, 368 (1911).
11. Bouveault, L., *Bull. Soc. chim.*, **15**, 1015 (1896).
12. Johnson, T. B., *J. Am. Chem. Soc.*, **61**, 2485 (1939).
13. Deulofeu, V., *Z. physiol. Chem.*, **204**, 214 (1932).
14. Boyd, W. J., and Robson, W., *Biochem. J.*, **29**, 546 (1935).
15. du Vigneaud, V., Chandler, J. P., Moyer, A. W., and Keppel, D. M., *J. Biol. Chem.*, **131**, 57 (1939).
16. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **37**, 572 (1919).
17. du Vigneaud, V., Kilmer, G. W., Rachele, J. R., and Cohn, M., *J. Biol. Chem.*, **155**, 645 (1944).
18. Chandler, J. P., and du Vigneaud, V., *J. Biol. Chem.*, **135**, 223 (1940).
19. Snell, E. E., Eakin, R. E., and Williams, R. J., *J. Am. Chem. Soc.*, **62**, 175 (1940).

SPECTROPHOTOMETRIC DETERMINATION OF SMALL AMOUNTS OF CHOLINE

By RICHARD J. WINZLER AND EMILY R. MESERVE*

(*From the Department of Biochemistry and Nutrition, University of Southern California Medical School, Los Angeles*)

(Received for publication, April 17, 1945)

Several investigators have assayed choline by precipitating it with Reinecke salt and determining colorimetrically the choline reineckate dissolved in acetone (1–6). The extinction coefficient of choline reineckate is not great in the visible range, however, and samples containing less than about 1 mg. of choline have been analyzed with difficulty. An increase in the sensitivity of the reineckate method has been achieved by oxidation of the precipitate with alkaline peroxide and determination of the chromate colorimetrically (7). A comparable increase in sensitivity can be obtained without this oxidation by making use of the very great absorption of ultraviolet light by choline reineckate. This is the basis of the method described in the present paper.

Spectrophotometry of Choline Reineckate—The absorption spectrum of choline reineckate in acetone was determined (Fig. 1) with a Beckman spectrophotometer. In addition to the familiar broad absorption band with a maximum at 525 mμ, choline reineckate in acetone was found to have a much more intense absorption peak at 327 mμ, the molecular extinction coefficients being 5.82×10^3 at 327 mμ, and 0.12×10^3 at 525 mμ. The Beer-Lambert law relating concentration and extinction is followed at both wave-lengths (Fig. 2).

Procedure

Samples to be analyzed are extracted with methanol in Bailey-Walker extractors for 24 hours. After evaporation of the methanol, 15 ml. of saturated barium hydroxide are added to the extracted lipids and refluxed for 2 hours. The hydrolysate is then brought to a pH between 4 and 5 with glacial acetic acid, made to volume, and filtered. An aliquot of the filtrate containing 50 to 400 γ of choline is treated with an equal volume of saturated recrystallized Reinecke salt, and the mixture is kept in an ice bath for at least 3 hours. Of the several methods investigated for the collection and washing of very small amounts of choline reineckate, the use of sintered glass filters kept chilled in an ice bath gave the best results in our hands. The precipitated choline reineckate is collected on the

* Best Foods Fellow in Biochemistry.

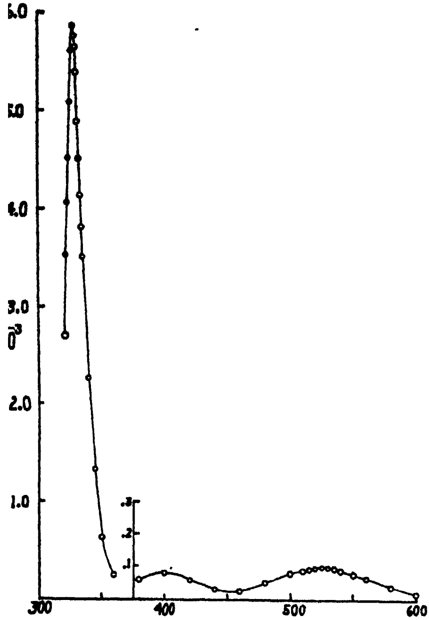


FIG. 1

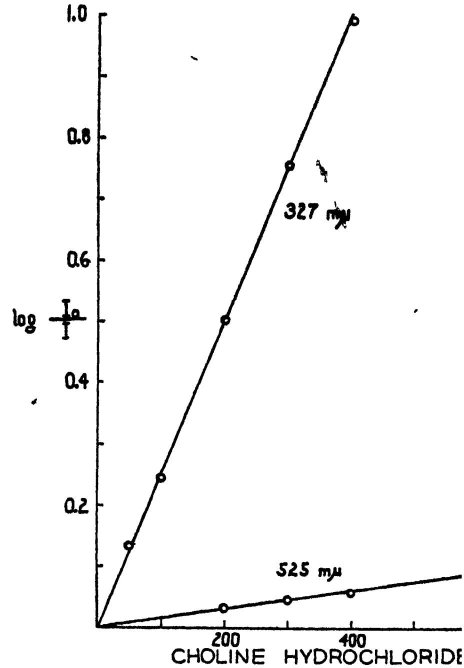


FIG. 2

FIG. 1. Absorption spectrum of choline reineckate in acetone. Molecular extinction coefficient plotted against wave-length.

FIG. 2. Relation between concentration and absorption. Choline hydrochloride was determined as indicated in the procedure and the absorption determined at 327 mμ and 525 mμ on the Beckman spectrophotometer. The values obtained at 525 mμ were in 5 ml. of acetone, while those at 327 mμ were in 15 ml. The choline hydrochloride concentration is given in micrograms.

TABLE I

Determination and Recovery of Choline in 10 Ml. Aliquots from 25 Ml. of Hydrolysate

Substance	Amount hydrolyzed	Choline HCl added	Choline HCl in aliquot	Choline HCl in sample		Recovery
	mg.	γ	γ	γ	γ *per gm.*	*per cent*
Blank, not extracted		0	0	0		
" " "		250	105	262		105
Crude lecithin, not extracted	5	0	74	187	37.4	
" " " "	5	250	170	425		96
Dried rat liver	25	0	122	305	12.2	
" " "	25	250	220	550		98
" yeast	100	0	148	370	3.70	
" "	100	250	244	610		96

chilled sintered glass by applying suction to the filter submerged in an ice bath. The precipitate, still in the ice bath, is washed with three 2 ml. portions of ice-cold water saturated with choline reineckate at 0°. Failure to wash with water saturated with choline reineckate leads to low recoveries, since choline reineckate is appreciably soluble in cold water. As much water as possible is then removed from the filter and precipitate by continued aspiration. This is essential, because water absorbs much less strongly than acetone at 327 mμ.

The precipitate is dissolved by passing 15 ml. of acetone through the sintered glass filter, and the optical density then determined at 327 mμ. Further dilution may be necessary if the sample contains more than 400 γ of choline. If the sample contains more than about 1000 γ of choline, readings can satisfactorily be made with a green filter and an ordinary photocolorimeter. The amount of choline in the sample can then be determined mathematically in the usual manner, or from calibration curves such as are shown in Fig. 2. The accuracy of this method, is, in the range of 50 to 400 γ of choline hydrochloride, about ± 5 per cent.

The results in Table I illustrate the applicability of the method to the determination of total choline in several materials. The recovery of choline added before extraction and barium hydroxide hydrolysis is seen to be satisfactory in all cases. The present method suffers from the same lack of specificity encountered with other reineckate methods, since a number of compounds forming insoluble reineckates are known. However, as pointed out by Engel (3), Glick (6), and others, such interfering substances are largely eliminated by methanol extraction and barium hydroxide hydrolysis. Here, as in other reineckate methods, only total choline is determined.

SUMMARY

An increase in the sensitivity of the colorimetric determination of choline as the reineckate is achieved by employing ultraviolet light instead of visible wave-lengths, choline reineckate in acetone having an intense absorption band with a peak at 327 mμ. The method has an accuracy of ± 5 per cent with samples containing 50 to 400 γ of choline hydrochloride.

BIBLIOGRAPHY

1. Beattie, F. J. R., *Biochem. J.*, **30**, 1554 (1936).
2. Jacobi, H. P., Baumann, C. A., and Meek, W. J., *J. Biol. Chem.*, **138**, 571 (1941).
3. Engel, R. W., *J. Biol. Chem.*, **144**, 701 (1942).
4. Thornton, M. H., and Broome, F. K., *Ind. and Eng. Chem., Anal. Ed.*, **14**, 39 (1942).
5. Entenman, C., Taurog, A., and Chaikoff, I. L., *J. Biol. Chem.*, **155**, 13 (1944).
6. Glick, D., *J. Biol. Chem.*, **156**, 643 (1944).
7. Marenzi, A. D., and Cardini, C. E., *J. Biol. Chem.*, **147**, 363 (1943).

SODIUM PHENOLPHTHALEIN PHOSPHATE AS A SUBSTRATE FOR PHOSPHATASE TESTS*

By CHARLES HUGGINS AND PAUL TALALAY

(*From the Department of Surgery, University of Chicago, Chicago*)

(Received for publication, March 16, 1945)

Most of the determinations of phosphatase activity have been made with three substrates, glycerophosphate introduced by Kay (1), phenyl phosphate by King and Armstrong (2), and to a much smaller extent with hexose phosphate (3). Specifically, α- and β-sodium glycerophosphates (4) and disodium and dipotassium (5) phenylphosphates have been utilized as substrates. We propose the use of a soluble phenolphthalein phosphate, water-clear until enzymic decomposition, when phenolphthalein is liberated and determined quantitatively in a colorimeter. The advantages of the new method are simplicity and accuracy; determinations are made without preliminary separation of proteins and the number of technical manipulations is small. Further, the kinetics of alkaline phosphatase activity may be followed continuously without sampling and a record of hydrolysis in relation to time obtained.

King (6) has synthesized several phosphoric esters which are colorless, or nearly so, and which liberate chromogen after phosphatase hydrolysis; among these are the calcium, lead, and barium salts of phenolphthalein phosphate. With these compounds King found that the "rate of enzymic hydrolysis is too slow and that too large amounts of plasma are required for it to constitute the basis of a quick and accurate procedure." This ingenious work of King is the basis of a qualitative test (7) for the production of phosphatase by certain genera of bacteria: a suspension of calcium phenolphthalein phosphate is incorporated in bacteriologic media upon which bacteria are inoculated; after growth for 18 hours or longer, ammonia vapor is blown over the culture with formation of a red color where phosphatase has been elaborated.

The calcium, lead, and barium salts of phenolphthalein phosphate are not readily soluble in water. In this laboratory the sodium salt was prepared and found to be freely soluble, colorless, and stable, but rapidly hydrolyzed by small quantities of phosphatase.

Synthesis of Sodium Phenolphthalein Phosphate

The method of preparation follows the customary procedure for making organic phosphates, with special precautions to obtain a compound free

* This investigation was supported by a grant from the Albert and Mary Lasker Foundation, Inc., and the Sidney and Frances Brody Foundation.

from uncombined phenolphthalein. Phenolphthalein is treated with phosphorus oxychloride in pyridine solution. The acid chloride is hydrolyzed to phenolphthalein diphosphoric acid, and the latter is converted into its sodium salt.

Method

For 0.1 M quantities, these directions may be followed. To 32 gm. of phenolphthalein is added fairly rapidly with mechanical stirring a mixture of 50 gm. (30 cc.) of pure, dry phosphorus oxychloride in about 50 cc. of dry chloroform. Dry pyridine, 25 cc., is then added slowly, while stirring rapidly and cooling the reaction mixture with ice. The addition of pyridine should be regulated so that the reaction flask does not become hot. The mixture should be stirred for several hours and should be allowed to stand at least overnight to permit the reaction to go to completion. The degree of completion of the reaction may be determined by withdrawing small samples of the reaction mixture and adding excess alkali. The intensity of the red color is a measure of the amount of free unchanged phenolphthalein and serves as an index of the state of completion of the reaction.

The reaction mixture is evaporated *in vacuo* at room temperature or with slight warming in order to remove most of the chloroform. About 100 cc. of water are then slowly added with stirring and cooling. There is copious evolution of hydrochloric acid, and a white precipitate forms. Strong sodium hydroxide (40 per cent) is added until all the precipitate redissolves and the solution is alkaline to phenolphthalein; the small amounts of phenolphthalein in the solution serve as its own indicator. The alkaline solution is extracted twice with ether in order to remove pyridine. If the solution is too viscous, some water may be added, although it is desirable to keep the volume as small as possible in order to facilitate the later precipitation of phenolphthalein diphosphoric acid. The aqueous layer is acidified with concentrated hydrochloric acid until blue to Congo red. This precipitates phenolphthalein diphosphoric acid, which is a glistening, viscid mass. When precipitation is approximately complete, the supernatant solution is decanted and the free acid dried *in vacuo* over calcium chloride. The dry, crude phenolphthalein diphosphoric acid is ground in a mortar to a white powder.

The powdered acid is dissolved in methanol, to which some pyridine has been added to increase the solubility. A solution of sodium ethoxide in ethanol, made by dissolving metallic sodium in absolute ethyl alcohol, is added until no further precipitation occurs. The sodium phenolphthalein phosphate separates as a copious white precipitate which is filtered off or the supernatant decanted. The precipitate is washed with repeated changes of alcohol and ether, until it is quite dry.

The preparation at this stage should be white and give almost no color in alkaline solution. It is repurified by dissolving in a mixture of methanol (80 parts by volume) and formamide (20 parts by volume), reprecipitating with absolute ethanol, and washing the precipitate with ethanol and ether. Reprecipitation from aqueous solution is not so satisfactory, since the sodium salt comes down as a sticky mass. It has not been possible to crystallize this compound in good yield; none of the preparations has contained free phenolphthalein, although inorganic phosphate is usually present in small amount. The sodium phenolphthalein phosphate should be kept dry, dark, and cold. Under these conditions it remains quite stable.

Analyses of the most pure preparations have shown sodium, 11.68 per cent; total phosphorus, 9.46 per cent (calculated for $C_{20}H_{13}O_{11}P_2Na_5$; sodium, 19.0 per cent, and phosphorus, 10.24 per cent). Although the preparation to date is not pure and contains some free inorganic phosphate, this does not affect its serviceability in determining phosphatase activity.

Principles Underlying Performance of Phosphatase Test—Phosphatase liberates from the colorless soluble ester the quinonoid structure on which the color of phenolphthalein depends, producing redness at alkaline pH in relation to the activity of the enzyme present; the color is intensified maximally, stabilized, and the enzyme inhibited by adding a buffer at an appropriate alkaline pH, containing a phosphatase inhibitor.

EXPERIMENTAL

Amount of Color of Phenolphthalein and Its Stability in Relation to pH—Phenolphthalein was dissolved in alcohol and diluted with water so that 5 mg. were contained in 1 liter; 5 cc. of this solution were mixed with 5 cc. of 0.25 M glycine sodium hydroxide buffer at varied pH which was measured electrometrically and the light transmission determined with a 540 mμ filter; the colorimetric reading was recorded immediately, and after 1 hour, at room temperature. Maximum color was developed at pH 10.3 to 11.4, but solutions more alkaline than pH 10.9 faded appreciably in 1 hour (Fig. 1).

Optimum pH for Acid and Alkaline Phosphatases with Phenolphthalein Phosphate As Substrate—The optimum rate of hydrolysis with varying pH for acid phosphatase in 0.2 N sodium acetate-acetic acid buffer at 37° was determined with human semen (1:100) as the source of enzyme; the amount of reaction product was measured as inorganic phosphate by the method of Fiske and Subbarow (8). The optimum activity was found to be between pH 5.5 and 6.0.

Similar determinations were made for alkaline phosphatase in 0.1 M sodium barbital-hydrochloric acid (9) and in 0.1 M sodium borate-sodium hydroxide (10) at varying pH with an extract of cattle intestinal mucosa

phosphatase. The optimum activity was between pH 9.1 and 9.6. The activity optima were plateaus in the ranges stated for acid and alkaline phosphatases respectively.

Inhibition of Phosphatase Activity—Practically it is necessary rapidly to suppress enzymic activity at an arbitrary time while measuring the reaction products; it is required further that the inhibitors do not precipitate proteins or interfere with the color. This may be done effectively by using an alkaline buffer containing sodium pyrophosphate which has been found to be an effective inhibitor of alkaline phosphatase (11). In the amounts stated in the test below, 0.25 M glycine-sodium hydroxide at pH 11.2 elevated a mixture of serum and acetate-buffered substrate

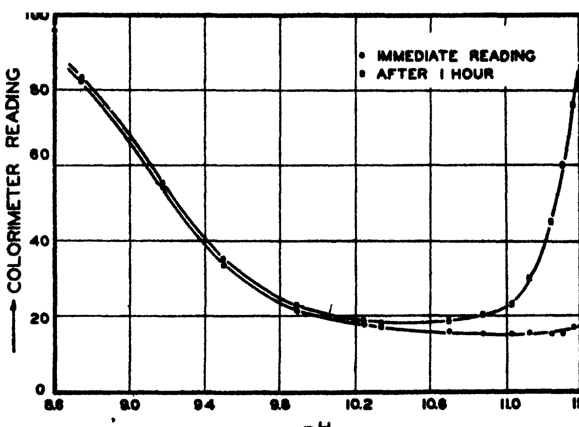


FIG. 1. Intensity and stability of phenolphthalein color at various pH in glycine-sodium hydroxide buffers. Colorimeter readings taken immediately on addition of buffer and after 1 hour.

to pH 10.6 with a stable color. The same glycine buffer introduced into the stated amounts of serum and barbital-buffered substrate increased the pH to 10.9 and produced a color which was stable for 1 hour.

Varying Concentrations of Substrate—With sodium phenolphthalein phosphate and a strong phosphatase preparation it was found that the rate of hydrolysis is independent of substrate concentration between 0.0005 M and 0.002 M. Therefore, 0.001 M sodium phenolphthalein phosphate was adopted in this work.

Kinetics of Hydrolysis and Definition of Units

In all quantitative phosphatase tests it is assumed that the amount of product formed under standard conditions is proportional to the enzyme

concentration, provided an excess of substrate is used. This was confirmed for phosphate liberated from sodium β-glycerophosphate and for phenol liberated from disodium monophenyl phosphate, in each case with a wide range of enzyme concentrations. In the case of the enzymic hydrolysis of sodium phenolphthalein phosphate, the relation between amount of phosphate liberated and enzyme concentration is entirely rectilinear, but the relation between phenolphthalein liberated as determined colorimetrically and the enzyme concentration is a parabolic curve.

The shape of the curve was determined for both acid and alkaline phosphatases with known dilutions with water of sera and seminal plasma containing high amounts of phosphatases; the same curves were obtained with known dilutions of human semen or serum as sources of acid phosphatase and also dilutions of an intestinal mucosa phosphatase preparation. The curves of phenolphthalein liberated relative to enzyme concentration were compared by defining the amount of enzyme which liberated 1 mg. of phenolphthalein in 1 hour as 10 units; for each experiment the relative enzyme concentrations were known from the dilutions which were made, and the enzyme concentrations in different experiments could then be compared by fixing the 10 unit point. In this manner, essentially identical parabolic curves were obtained for amount of product *versus* enzyme concentration for twelve different preparations of acid and alkaline phosphatases.

The reason for this discrepancy between the amount of phosphorus liberated and the amount of phenolphthalein as determined colorimetrically is not known. It is probably due to the existence of two hydrolytic products of phenolphthalein diphosphate.

Analysis of the curve of color density *versus* enzyme concentration revealed that it is a strict parabola which can be resolved into a linear graph by a plot of the log of color density expressed as mg. of phenolphthalein against the log of the enzyme concentration. This curve is strictly linear over the entire range of enzyme concentrations investigated (Fig. 2). The slope of the curve relating the amount of phenolphthalein liberated and the enzyme concentration does not depend on the time of incubation between 30 and 120 minutes (Fig. 3).

The linearity of the log-log plot enables the use of convenient units. We define *10 units of acid or alkaline phosphatase as the amount of enzyme which will liberate the colorimetric equivalent of 1 mg. of phenolphthalein from excess substrate in 1 hour at 37° under optimum conditions of pH.*

It is important to remember that there is not a linear relation between the units of phosphatase and the amount of phenolphthalein liberated for the reasons explained above.

In order to obtain the unitage, a log-log plot is made of log mg. of phenol-

phthalein liberated by 100 cc. of serum *versus* log enzyme concentration. The reference point of the plot is 1 mg. of phenolphthalein related to 10

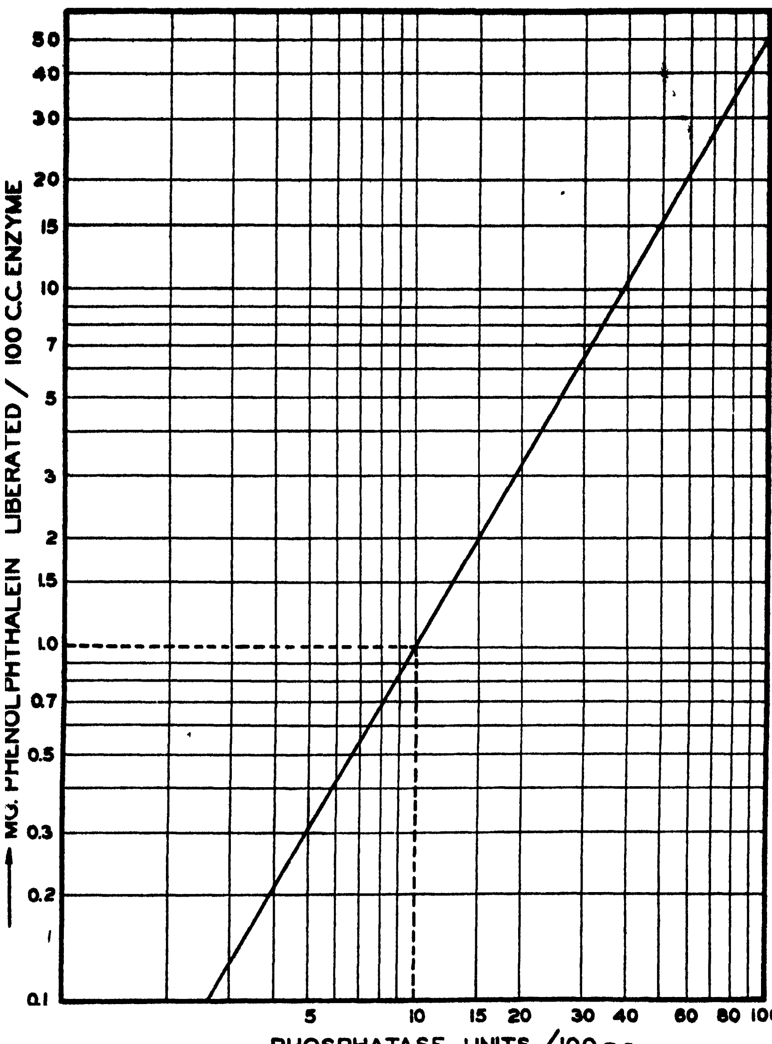


FIG. 2. Log-log plot of mg. of phenolphthalein liberated by 100 cc. of serum against enzyme concentration in units per 100 cc. By definition of units 1 mg. of phenolphthalein is liberated by 10 units of enzyme. Composite plot of twelve experiments with various preparations of acid and alkaline phosphatases.

units of enzyme. The plot is a straight line (Fig. 2), the slope of which may be conveniently determined experimentally as follows. A phosphatase

preparation of fairly high activity, *e.g.* a pathological serum, or urine from a normal young adult human male, is diluted to give a number of relative enzyme concentrations. These are run through the test and the amount of mg. of phenolphthalein liberated by 100 cc. of enzyme preparation is computed. A plot is made of mg. of phenolphthalein *versus* the enzyme

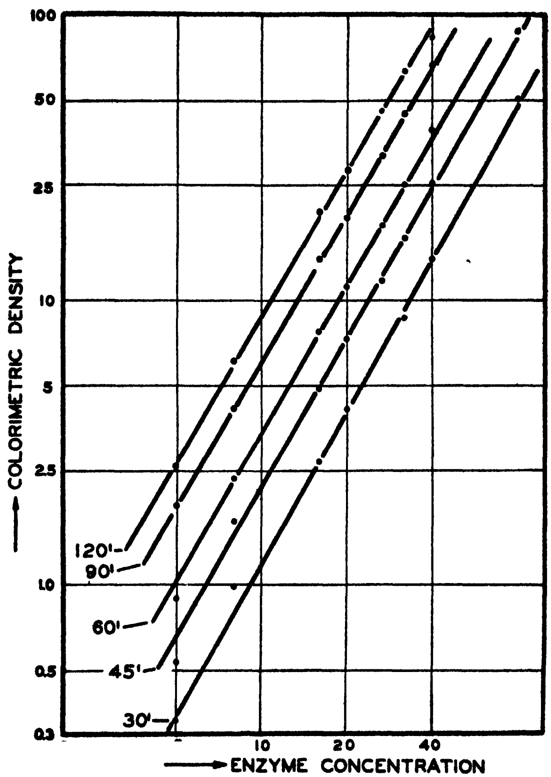


FIG. 3. Log-log plot of phenolphthalein liberated, expressed as colorimeter density and enzyme concentration in relative units at various times of incubation. 9 cc. of 0.001 M sodium phenolphthalein phosphate in sodium barbital buffer (20.6 gm. per liter) plus 1 cc. of cattle intestinal mucosa phosphatase. Incubated at 37°, color read at 30, 45, 60, 90, and 120 minutes in an Evelyn colorimeter.

concentration in arbitrary units on log-log graph paper (three cycles by two cycles will be found convenient). The points will lie on a straight line. The unit curve which is being determined is then a line parallel to the line found above, but passing through the point of definition of our unit, *i.e.* 1 mg. = 10 units. The equation relating P, the number of mg. of phenolphthalein liberated by 100 cc. of enzyme solution, and U, the

number of units of phosphatase per 100 cc. of enzyme solution, is of the form, $\log U = K \log P + C$ in which K and C are constants. If 10 units liberate 1 mg. of phenolphthalein in 1 hour, $C = 1.00$, and the slope of the graphical plot gives the equation, $\log_{10} U = 1.00 + 0.581 \log_{10} P$. The unit curve can be determined simply in any laboratory. For convenience the data of this curve may be compiled in a table, from which units can be read, if the amount of phenolphthalein liberated by 100 cc. of enzyme preparation is known.

If for any reason it becomes desirable to use an incubation time different from 1 hour, say 2 hours, and the unit of phosphatase activity is then defined as 10 units liberating 1 mg. of phenolphthalein in 2 hours, the same table and curve may be employed.

Reagents—

Acid-buffered substrate solution. 11.70 gm. of sodium acetate, $NaC_2H_3O_2 \cdot 3H_2O$, and 0.79 cc. of 99 per cent glacial acetic acid are dissolved in distilled water, 0.608 gm. of sodium phenolphthalein phosphate is added, and the mixture diluted to 1 liter. As a preservative 7.5 cc. of chloroform are added and the reagent is stored in a refrigerator. The solution is about 0.001 M with respect to the substrate and has a pH of 5.4.

Alkaline-buffered substrate solution. Mix 20.6 gm. of sodium barbital and 0.608 gm. of sodium phenolphthalein phosphate in distilled water and dilute to 1 liter. Add 7.5 cc. of chloroform. The pH of the solution is 9.7.

Glycine buffer. A concentrated carbonate-free sodium hydroxide solution is prepared by adding carefully 100 gm. of sodium hydroxide with stirring to 100 cc. of water in a beaker immersed in water. Dissolve 9.19 gm. of glycine (aminoacetic acid) and 7.17 gm. of sodium chloride in water; add 15 cc. of the concentrated sodium hydroxide solution and water to nearly 1 liter; then add 40 gm. of powdered sodium pyrophosphate ($Na_4P_2O_7 \cdot 10H_2O$) and dilute to the mark. The pH is 11.2.

Stock phenolphthalein solution. Dissolve 100 mg. of phenolphthalein in 100 cc. of 95 per cent ethyl alcohol; keep well stoppered.

Procedure

The test is performed in well matched colorimeter tubes which are washed until they are scrupulously free from acids or alkalies used in cleaning. The rubber stoppers are boiled and freed from "bloom," since this contaminant has acted as a phosphatase inhibitor. A photoelectric colorimeter with a light source filtered to 540 mμ wave-length was used throughout.

Phenolphthalein Calibration Curve—Dilutions of stock phenolphthalein solution are made, for example 2, 4, and 10 cc. per liter of distilled water. 5 cc. of each dilution are placed in a colorimeter tube and 5 cc. of glycine

buffer for color development for phosphatase tests. The color density is determined *immediately* after mixing in a colorimeter. The density is plotted against mg. of phenolphthalein per liter on graph paper and the points fall on a straight line.

Phosphatase Tests—As a blank control 0.5 cc. of test solution is added to 5 cc. of distilled water and 4.5 cc. of glycine buffer and the density read in the colorimeter. The same tube serves as a blank for both acid and alkaline phosphatase determinations.

Colorimeter tubes in duplicate or triplicate, each containing 5 cc. of the appropriate buffered substrate (acid or alkaline), are equilibrated in a water bath at 37° for about 5 minutes. The test solution is also warmed and 0.5 cc. is added to each tube which is rotated for mixing; it is advisable not to invert the solution. The mixture is incubated for precisely 1 hour, when 4.5 cc. of glycine buffer are added and the color density read immediately. The light transmission for the corresponding control tube is previously adjusted to read zero on the colorimeter scale (100 on the Evelyn type colorimeter). The reading of the experimental tube measures the density of the color produced in the test. It is necessary to dilute the color in sera with high activity. A 4-fold dilution may be achieved in the colorimeter tubes (170 × 20 mm.), but if the color is very intense 5 cc. of the solution are diluted with water to 50 cc. or 100 cc. in a volumetric flask, and the color is read again against an equally diluted control. The dilution of the buffered solution with distilled water does not produce an appreciable pH effect on the color intensity.

The pH of the system, serum and acetate-buffered substrate, is 5.6, and after adding glycine buffer is 10.6. The pH of the system, serum and barbital-buffered substrate, is 9.6, and after adding glycine is 10.9.

Calculation of Results—The colorimeter reading of the test solution is converted to equivalent phenolphthalein concentration in mg. per liter, by means of the standard calibration curve.

Mg. phenolphthalein liberated by 100 cc. of serum = observed concentration of phenolphthalein in mg. per liter ×

$$\frac{10 \text{ cc.}}{1000 \text{ cc.}} \times \frac{100 \text{ cc. serum}}{0.5 \text{ cc. serum}} =$$

2 × observed concentration of phenolphthalein in mg. per liter

in which 10 cc. are total volume of test and 1000 cc. represent the volume of phenolphthalein standard.

The density obtained in mg. of phenolphthalein per liter is therefore multiplied by 2, and the equivalent units of phosphatase per 100 cc. are read directly from Fig. 2 or a similarly constructed graph.

All calculation can be eliminated if the phenolphthalein calibration

curve is modified by plotting twice the phenolphthalein concentration per liter against the colorimeter reading.

Results

Accuracy—In routine testing the accuracy of duplicate checks is within ±2 per cent for normal human sera.

Serum—The phosphatase levels of fifty-six normal adult humans (forty-one males aged 21 to 65 and fifteen females aged 21 to 50) were determined. For acid phosphatase, the average is 5.9 units per 100 cc. with a range of 3 to 10 units and the peak of the distribution curve lies between 4.0

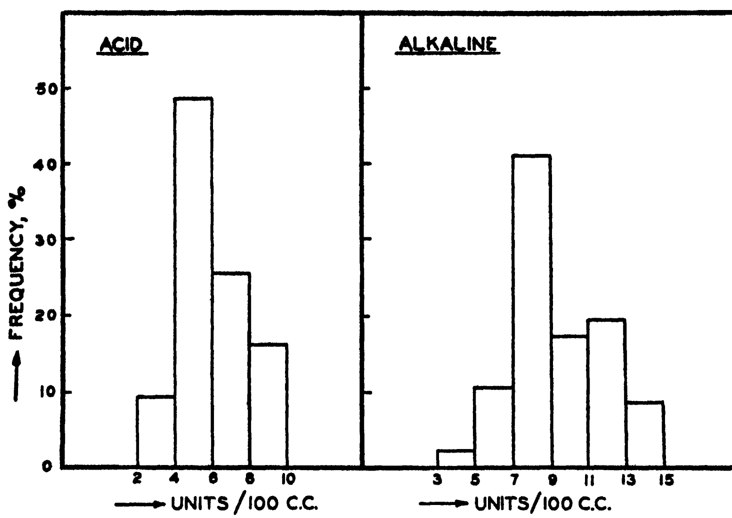


FIG. 4. Distribution curves of normal human serum phosphatase values of fifty-six individuals of both sexes. Acid phosphatase average, 5.9 units per 100 cc. Alkaline phosphatase average, 9.5 units per 100 cc.

and 6.0 units (Fig. 4). For alkaline phosphatase, the numerical average is 9.5 units per 100 cc. with a range extending from 3 to 15 units with a distribution curve peak at 7.0 to 9.0 units (Fig. 4). No difference between the sexes could be demonstrated in the sera on this small sample.

In these laboratories 1 hour has been found satisfactory for most determinations on biological materials; 2 hours of incubation produce more accurate results for very low phosphatase values. The sera of fifty-nine normal persons (thirteen females aged 21 to 58 and forty-six males aged 17 to 76) gave a mean average; for acid phosphatase 0.66 mg. and for alkaline phosphatase 2.57 mg. of phenolphthalein were liberated per 100 cc. of serum in 2 hours.

Milk—In testing cow's milk, this fluid should be diluted with an equal quantity of water to facilitate transmission of light through the samples. Six samples of raw milk contained the following phosphatase values in units per 100 cc.; acid phosphatase 6.1 to 10.0 units; alkaline phosphatase 46.2 to 84.0 units. Kay and Graham (12) have previously described the effects of pasteurization on phosphatases in milk.

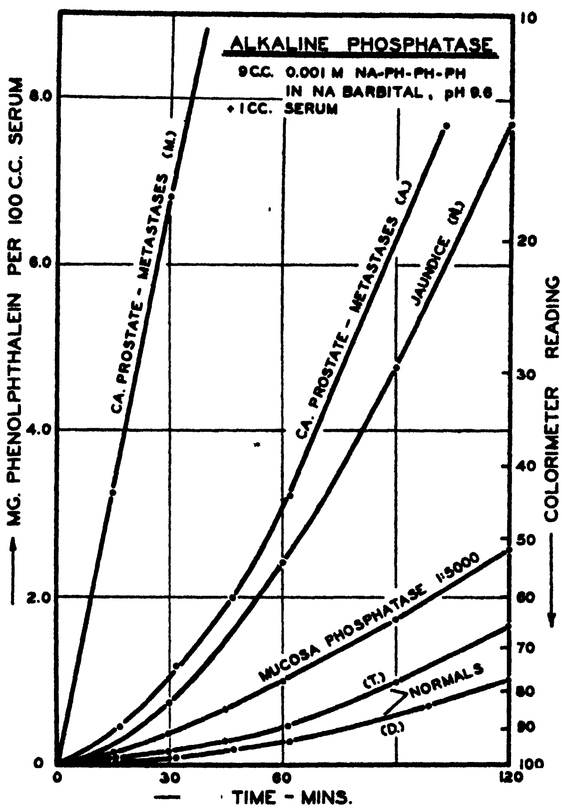


FIG. 5. Continuous study of alkaline phosphatase hydrolyses. Graphs obtained with three pathological and two normal human sera; also a dilute cattle mucosa phosphatase preparation. Incubation at 37°. Color read in an Evelyn colorimeter (540 mμ filter).

Urine—Freshly voided urine of six young normal adult human males had the following values per 100 cc.; acid phosphatase 38 to 56.4 units; alkaline phosphatase 1.5 to 5.0 units. In a previous study of urinary phosphatases (13) with the method of King and Armstrong (2) it was found necessary to dialyze the urine to eliminate interfering chromogens. Dialysis is not necessary with the present method.

Continuous Study of Kinetics of Alkaline Phosphatases

Since the optimum pH of alkaline phosphatase is more alkaline than the "end-point" of phenolphthalein (about pH 8.3), the progress of the enzymic hydrolysis can be observed continuously by reading the color intensity with respect to time. A colorimeter of the Evelyn type was mounted in a 37° incubator, the galvanometer being outside where it was observed conveniently. The usual alkaline-buffered substrate was used, but no glycine was added. The reaction occurred directly in a colorimeter tube which was left in the colorimeter, and increases of density were observed continuously (Fig. 5).

SUMMARY

The synthesis of sodium phenolphthalein phosphate is described. The compound is easily soluble in water, contains no free phenolphthalein but, to date, is impure, being contaminated with a small amount of inorganic phosphate; it is, however, serviceable as a substrate for phosphatase tests.

In phosphatase determinations with this substrate, color is directly measured without precipitation of proteins and with few technical operations; the accuracy is within ±2 per cent in duplicates. In addition to the convenience and accuracy of the method, the kinetics of alkaline phosphatase activity can be studied directly and continuously without sampling.

BIBLIOGRAPHY

1. Kay, H. D., *J. Biol. Chem.*, **89**, 235 (1930).
2. King, E. J., and Armstrong, A. R., *Canad. Med. Assn. J.*, **31**, 376 (1934).
3. Levene, P. A., and Dillon, R. T., *J. Biol. Chem.*, **88**, 753 (1930).
4. Bodansky, A., *J. Biol. Chem.*, **101**, 93 (1933).
5. Kutscher, W., and Wolbergs, H., *Z. physiol. Chem.*, **236**, 237 (1935).
6. King, E. J., *J. Path. and Bact.*, **55**, 311 (1943).
7. Bray, J., and King, E. J., *J. Path. and Bact.*, **55**, 315 (1943).
8. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
9. Michaelis, L., *J. Biol. Chem.*, **87**, 33 (1930).
10. Kolthoff, I. M., and Vleeschhouwer, J. J., *Biochem. Z.*, **189**, 191 (1927).
11. Schmidt, G., and Thannhauser, S. J., *J. Biol. Chem.* **149**, 369 (1943).
12. Kay, H. D., and Graham, W. R., Jr., *J. Dairy Res.*, **6**, 191 (1935).
13. Scott, W. W., and Huggins, C., *Endocrinology*, **30**, 107 (1942).

THE CHEMISTRY OF BONE FORMATION

I. THE COMPOSITION OF PRECIPITATES FORMED FROM SALT SOLUTIONS

By ADRIAN C. KUYPER*

(*From the Department of Biochemistry, Tulane University, New Orleans*)

(Received for publication, December 30, 1944)

In a number of respects the composition of calcified tissue has been found to be similar to a precipitate formed from salt solutions supersaturated with respect to calcium phosphate. Both bone and precipitated basic calcium phosphate give similar x-ray diffraction patterns characteristic of the apatite structure (1). Carbonate is present in bone and in the precipitate formed from salt solutions even when the calcium carbonate solubility product is not exceeded (2). The substance which is first formed both in calcification and in precipitation from salt solution has been thought to be a dicalcium phosphate which is rapidly changed to a basic calcium phosphate (3). Adsorption and hydrolytic reactions appear to cause similar changes in bone structure and in calcium phosphate precipitates (2, 4). Differences between the inorganic composition of calcified tissues of the same animal, as well as their solubility behavior, have been attributed to the action of the tissue cells (5, 6).

The experiments reported in this paper were performed with the purpose of determining the equivalent composition of precipitates formed from serum salt solutions in order that the precipitates might be more closely compared with bone. It was found that their composition varies with the composition of the solutions with which they are in contact. When in contact with a solution of the same salt composition as blood serum the precipitate has approximately the composition of bone or dentin. When in contact with a solution of the same salt composition as saliva, the precipitate has nearly the same composition as enamel.

EXPERIMENTAL

The precipitates which were analyzed were formed from solutions prepared by measuring out and dissolving in water the required amounts of potassium acid phosphate, calcium chloride, magnesium chloride, sodium bicarbonate, sodium chloride, and sodium citrate. In order that precipitates might form at physiological pH it was necessary that calcium and phosphate be present in excess of the amount present in the diffusible

* Present address, Department of Physiological Chemistry, Wayne University College of Medicine, Detroit.

forms in blood serum. Sufficient sodium bicarbonate and sodium chloride were used to bring the bicarbonate content and ionic strength of the solutions after precipitation to the composition of blood. To prevent precipitation immediately upon the addition of sodium bicarbonate, the solutions were presaturated with carbon dioxide gas. The solutions were placed in a constant temperature bath at 38° and stirred continuously by bubbling through them a mixture of carbon dioxide and air. The pH was controlled by the amount of carbon dioxide in the gas mixture. Precipitation occurred within 3 to 8 hours.

The gas mixtures used in equilibration were prepared from air and carbon dioxide passed through capillary flow tubes. The pressures across the capillary tubes and the composition of the mixtures were readily controlled by allowing excess gases going into and leaving the capillaries to escape under suitable heads of water. Gases were saturated with water vapor at 38° before they were passed through the experimental solutions.

At the end of the period of equilibration the precipitates were allowed to settle, the supernatant solutions were siphoned off, and the residues were transferred to centrifuge tubes and immediately separated by centrifugation and decantation. The wet precipitates were weighed and analyzed. Analytical values for the dry precipitates were obtained by correcting for the supernatant solution present in the wet precipitates.

The carbonate present in the wet precipitates was determined by aeration in a closed circuit apparatus into a measured excess of sodium hydroxide solution, precipitation with strontium chloride, and titration of the excess sodium hydroxide with standard potassium acid phthalate (7). The solutions obtained after removal of carbon dioxide were diluted to volume and used for further analyses. Calcium was precipitated as the oxalate from solutions previously neutralized with sodium acetate, washed twice with ammonia solution saturated with calcium oxalate, and titrated with permanganate (8). The results were corrected for the coprecipitation of small amounts, 2 per cent, of sodium oxalate (9). Phosphorus determinations (10) checked within 1 per cent. Repeated determinations of the ratio of calcium to phosphorus in samples of primary and secondary phosphate were within 2 per cent of the theoretical. Citrate was determined by precipitation with calcium phosphate (11), oxidation to acetone, and iodometric determination of the acetone (12). The details of this procedure have not been published. Magnesium was precipitated as magnesium ammonium phosphate (13) and determined colorimetrically (10). The pH was calculated from the composition of the gas mixture used in aeration and the bicarbonate present in the solution (14).

Results

Table I gives the results of these experiments. The amount of carbonate in the precipitates varies with the amount of bicarbonate in the supernatant solutions (Experiments 1 and 2). It also varies inversely with the total amounts of calcium and phosphate in the supernatant solutions (Experiments 3 to 6, and 21 to 27). An increase in the ratio of calcium to phosphorus in the supernatant solution (Experiments 7 to 12) is accompanied by an increase in the amount of carbonate in the precipitate, but this might be attributed to a decrease in the total equivalent amount of calcium and phosphate (figures not shown) rather than to a change in the ratio of calcium to phosphorus. Solutions with the same calcium to phosphorus ratio (Experiments 3 to 5, figures not shown) may be associated with precipitates of widely different carbonate content. A decrease in either the amount of calcium with the phosphorus remaining constant (compare Experiments 4 and 23 with Experiment 7), or a decrease of phosphorus with the calcium remaining approximately constant (compare Experiments 6 and 12), is accompanied by an increase in the amount of carbonate in the precipitate. Change in pH produced by change in partial pressure of carbon dioxide appears to have no pronounced effect on the composition of the precipitate (Experiments 13 and 14). Citrate, which has previously been reported to be precipitated under somewhat similar conditions (11), is also precipitated under the conditions of these experiments. The amount precipitated varies with the amount present in the supernatant solution (Experiments 15 to 17). The presence of physiological concentrations of magnesium in the precipitating solution markedly decreases the rate of precipitation of the calcium phosphate (Experiments 18 to 27). The amount of magnesium carried down in the precipitate varies with the amount of magnesium and the amounts of calcium and phosphate present in the supernatant solution. In all the experiments and especially in those in which equilibration was carried on for a short time in the presence of magnesium (Experiments 21, 22, and 28), the anions of the precipitate exceed the cations. This may be attributed partly to the inclusion of from 2 to 3 per cent of the total cations as sodium and potassium and also to precipitation of some of the phosphate as dicalcium phosphate (2).

DISCUSSION

In order that the composition of these precipitates might be compared with the composition of bony tissue, typical analyses of bone, dentin, and enamel reported in the literature were calculated to the basis of equivalent ratios and recorded in Table II. The composition of bone and dentin varies considerably, but in general it corresponds to the precipitates in

TABLE I

Composition of Precipitates Formed from Salt Solutions

Experiment No.	Days of equilibration	Composition of solutions, mg. per 100 cc.								Composition of ppt. expressed as equivalent ratios (Ca = 100)			
		Before pptn.*		After pptn.									
		Ca	P	Ca	P	Mg	Citrate	Na-HCO_3	pH	P	CO_2	Mg	Citrate
1	3	13	6.5	3.0	2.2			420	7.5	85	20.0		
2	3	13	6.5	2.7	1.7			109	7.5	88	13.3		
3	0.2	18	8.0	14.4	6.2			210	7.4	97	6.5		
4	0.3	18	8.0	9.9	4.3			210	7.4	91	11.7		
5	0.6	18	8.0	4.7	2.0			210	7.4	87	14.8		
6	1.0	18	8.0	3.9	2.0			210	7.4	88	15.5		
7	3	10	8.0	1.4	4.0			210	7.5	91	14.0		
8	3	11	7.5	1.6	3.1			210	7.5	92	15.1		
9	3	12	7.0	1.7	2.3			210	7.5	90	15.7		
10	3	13	6.5	2.3	1.8			210	7.5	88	16.2		
11	3	14	6.0	3.2	1.3			210	7.5	87	18.0		
12	3	15	5.5	4.1	0.9			210	7.5	81	19.5		
13	4	12	7.0	1.3	2.2			210	7.7	89	17.4		
14	4	17	9.0	2.7	2.5			210	7.1	91	14.9		
15	3	17	7.0	4.2	1.5		1.5	210	7.4	84	16.5		1.8
16	3	13	11.0	1.1	5.4		0.8	210	7.4	91	13.0		1.3
17	3	17	7.0	5.8	2.1		3.4	210	7.4	88	15.4		3.4
18	3	17	7.0	11.0	3.9	2.3		210	7.4	101	9.1	5.5	
19	3	13	11.0	7.0	7.6	2.3		210	7.4	107	6.7	6.7	
20	3	17	7.0	11.7	4.0	4.8		210	7.4	110	7.9	9.9	
21	1	18	8.0	14.5	6.3	2.6	2.6	210	7.4	108	7.0	6.1	1.1
22	2	18	8.0	12.9	5.3	2.6	2.7	210	7.4	105	7.6	5.6	1.1
23	3	18	8.0	9.9	3.9	2.5	2.3	210	7.4	98	10.5	5.2	1.1
24	2	15	8.5	5.6	3.9	2.4	0.7	210	7.4	95	11.4	5.5	0.7
25	3	15	8.5	3.3	2.7	2.4	2.3	210	7.4	93	14.0	4.8	1.9
26	4	18	8.0	5.4	2.1	2.5	1.3	210	7.4	91	14.8	4.0	1.8
27	6	18	8.0	4.7	1.8	2.5	2.1	210	7.4	91	15.5	4.0	2.2
28	0.2	14	19.0	3.0	12.8	0.5		80	7.0	108	3.6	1.8	
29	0.7	14	19.0	2.2	12.5	0.5		80	7.0	103	4.5	1.6	
30	2.5	14	19.0	1.3	12.1	0.5		80	7.0	101	5.6	1.8	

* In addition, sodium bicarbonate and sodium chloride were present in all solutions. Magnesium and citrate were present only in those solutions which show analytical values for these substances. Citrate was slowly decomposed during the period of equilibration. It was present initially in amounts ranging from 4 to 8 mg. of citric acid per 100 cc. of solution. To those solutions equilibrated for more than 2 or 3 days it was added daily in an amount equivalent to 2 mg. of citric acid per 100 cc. of solution.

contact with solutions containing about 3.5 mg. of phosphorus and 5.5 mg. of calcium per 100 cc. of solution (Experiments 23 to 26, Table I). These are normal values of diffusible blood phosphorus and calcium (21); ionic

calcium and phosphorus are somewhat lower. Variations in the composition of bone and dentin appear to be of such order of magnitude that they might be attributed to variations in the inorganic composition of the blood. The presence of a smaller amount of carbonate in the bones of young than of old animals (17) may be related to the larger amount of calcium and phosphorus in the blood of the young animals.

Dental enamel contains proportionately less magnesium, carbonate, and citrate than dentin or bone. This appears to be related to the presence in saliva of smaller amounts of these substances than in serum. Saliva contains only 0.5 mg. of magnesium per 100 cc., about 80 mg. of sodium bicarbonate, and from zero to a few tenths of a mg. of citric acid (22–25). Although saliva contains very much more phosphorus, 15 mg. per 100 cc., than does serum, dental enamel contains about the same amount of phosphorus as dentin or bone. Precipitates formed from solutions of the inorganic composition of saliva (Experiments 28 to 30, Table I)

TABLE II

Composition of Calcified Tissues

Tissue	Bibliographic reference No.	Composition expressed as equivalent ratios (Ca = 100)							
		P		CO_2		Mg		Citrate	
		Range	Average	Range	Average	Range	Average	Range	Average
Bone	(15–17)	81–98	89	9.5–14.3	12.4	1.7–5.0	3.1	0.5–2.5	1.4
Dentin	(17–20)	94–97	95	10.4–12.4	11.3	5.0–5.3	5.2		0.9
Enamel	(17–20)	90–95	92	4.8– 7.7	6.5	1.1–2.1	1.5		0.08

have the same composition as enamel except for a somewhat higher phosphate and a lower carbonate content. The smaller phosphate content of enamel might be attributed to a hydrolysis such as occurs when calcium phosphate precipitates are allowed to stand in water for a long period of time (26). Close comparison between the composition of these precipitates and enamel appears to be unprofitable because of the rapid rate at which equilibrated solutions change composition and because of variations in the composition of saliva. The relationships which exist between the composition of these precipitates and the salt solutions with which they are in contact suggest that the integrity of tooth structure is dependent on the composition of the saliva.

SUMMARY

When calcium phosphate is precipitated from salt solutions under physiological conditions of pH, temperature, and ionic strength, the

composition of the precipitate varies with the composition of the precipitating solution. When calcium, magnesium, phosphate, bicarbonate, and citrate are present in the supernatant solution in the amounts in which they are present in serum ultrafiltrate, the precipitate contains these substances in approximately the same molecular ratios in which they are present in bone or dentin. When these substances are present in the supernatant solution in the amounts in which they are present in saliva, the precipitate contains them in roughly the same molecular ratios in which they are present in enamel.

BIBLIOGRAPHY

1. Taylor, N. W., and Sheard, C., *J. Biol. Chem.*, **81**, 479 (1929).
2. Logan, M. A., and Taylor, H. L., *J. Biol. Chem.*, **125**, 377, 391 (1938).
3. Shear, M. J., and Kramer, B., *J. Biol. Chem.*, **79**, 125 (1928).
4. Kramer, B., and Shear, M. J., *J. Biol. Chem.*, **79**, 147 (1928).
5. Murray, M. M., *Biochem. J.*, **30**, 1567 (1936).
6. McLean, F. C., and Hastings, A. B., *J. Biol. Chem.*, **108**, 285 (1935).
7. Kuyper, A. C., and Jones, L. M., *Ind. and Eng. Chem., Anal. Ed.*, **13**, 801 (1941).
8. Stanford, R. V., and Wheatley, A. H. M., *Biochem. J.*, **19**, 710 (1925).
9. Sendroy, J., Jr., *J. Biol. Chem.*, **152**, 539 (1944).
10. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
11. Kuyper, A. C., *J. Biol. Chem.*, **123**, 405 (1938).
12. Taufel, K., and Mayr, F., *Z. anal. Chem.*, **93**, 1 (1933).
13. Denis, W., *J. Biol. Chem.*, **52**, 411 (1922).
14. Hastings, A. B., Murray, C. D., and Sendroy, J., Jr., *J. Biol. Chem.*, **71**, 723 (1926-27).
15. Dickens, F., *Biochem. J.*, **35**, 1011 (1941).
16. Marek, J., Wellman, O., and Urbanyi, L., *Z. physiol. Chem.*, **226**, 3 (1934).
17. Logan, M. A., *J. Biol. Chem.*, **110**, 375 (1935).
18. Bowes, J. H., and Murray, M. M., *Biochem. J.*, **29**, 2721 (1935).
19. Armstrong, W. D., and Brekhus, P. J., *J. Biol. Chem.*, **120**, 677 (1937).
20. Free, A. E., *J. Dent. Res.*, **22**, 477 (1934).
21. Schmidt, C. L. A., and Greenberg, D. M., *Physiol. Rev.*, **15**, 297 (1935).
22. Krasnow, F., *J. Am. Dent. Assn.*, **25**, 216 (1938).
23. Karshan, K., *J. Dent. Res.*, **18**, 395 (1939).
24. Pucher, G. W., Sherman, C. C., and Vickery, H. B., *J. Biol. Chem.*, **113**, 235 (1936).
25. Kuyper, A. C., and Mattill, H. A., *J. Biol. Chem.*, **103**, 51 (1933).
26. Lorah, J. R., Tartar, H. V., and Wood, L., *J. Am. Chem. Soc.*, **51**, 1097 (1929).

THE CHEMISTRY OF BONE FORMATION

II. SOME FACTORS WHICH AFFECT THE SOLUBILITY OF CALCIUM PHOSPHATE IN BLOOD SERUM

By ADRIAN C. KUYPER*

(From the Department of Biochemistry, Tulane University, New Orleans, and the Laboratory of Physiological Chemistry, The Ohio State University, Columbus)

(Received for publication, December 30, 1944)

With respect to their physiological behavior, the inorganic calcium and phosphate of blood appear to be in equilibrium with the bone salts. When the product of the concentrations of these two ions exceeds a certain value, bony tissue becomes more highly calcified; when it is less, decalcification takes place. When the concentration of one of these ions is either raised or lowered, the other changes reciprocally (1).

This state of equilibrium has been only imperfectly reproduced, if at all, in inorganic salt solutions. Measurements of the solubility of calcium phosphate carried out in the presence of small amounts of the solid give values which vary from an extremely low solubility (2) to solubility equivalent to the calcium and phosphate present in the diffusible form in blood plasma (3). Since there is no adequate evidence to the contrary, these widely divergent values might be attributed to failure to reach true equilibrium. The most reliable measurements of the solubility of calcium phosphate, or of bone, have been made by the use of relatively large amounts of solid for equilibration. Solubility values obtained in this way by different investigators (4, 5) are not in good agreement but they amply demonstrate that the amounts of dissolved calcium and phosphate are much less than the amounts present in the diffusible forms in blood. This apparent difference in solubility is too large to be attributed entirely to the presence in blood of undissociated compounds such as calcium citrate (6) or creatine phosphate (7).

The experiments reported in this paper represent the results of about a thousand determinations of the solubility of calcium phosphate performed with the purpose of reconciling the apparent difference in the solubility of calcium phosphate in salt solution and in blood serum. They show that reliable solubility measurements are obtained only when large amounts of solid phase are present in the equilibrium mixtures. They also show that precipitates of calcium phosphate formed from inorganic solutions vary considerably in solubility, in general being most soluble when first formed from dilute solutions. Citrate, magnesium, and pyrophosphate

* Present address, Department of Physiological Chemistry, Wayne University College of Medicine, Detroit.

further increase this solubility. Precipitates formed from solutions containing citrate, magnesium, bicarbonate, and chloride, in the amounts present in blood, have for a few days following their formation a solubility which corresponds roughly to the inorganic calcium and phosphate of blood serum.

EXPERIMENTAL

The procedure for the preparation of the calcium phosphate precipitates and the methods of analysis were essentially the same as in Paper I (8). Solubility measurements were made at 38° in solutions which contained about 0.025 M sodium bicarbonate and sufficient sodium chloride to bring the ionic strength to 0.158. During the period of equilibration, solutions were shaken mechanically with an undulating motion as a carbon dioxide-air mixture was bubbled continuously through them. Samples for analysis were siphoned off through a filter system which did not allow contact of the solutions with air.

Pyrophosphate was determined by hydrolyzing a 5 or 10 cc. sample with 2.5 cc. of 2 N H_2SO_4 in a boiling water bath for 15 minutes, allowing the solution to cool to room temperature, and continuing with the determination of orthophosphate. Additional orthophosphate formed during the hydrolysis represents pyrophosphate. The solubility product constant of tricalcium phosphate was calculated by means of the constants and formulae developed by Sendroy and Hastings (4) and used as a measure of the solubility of the precipitates.

Influence of Amount of Solid Phase on Solubility of Calcium Phosphate (Table I)—These experiments were designed to investigate the contradictory results obtained by other workers (2, 3) and to establish an experimental basis for measuring the solubility of calcium phosphate precipitates. Calcium phosphate precipitate was added in increasing amounts to saline solutions which had been previously equilibrated at 38° with a mixture of carbon dioxide and air. The systems were then equilibrated for 15 days. To obtain evidence as to whether or not equilibrium was reached the composition of the gas mixture used in aeration was changed at the end of the 13th day of equilibration so that the pH was either decreased 0.09 unit (Experiments 1 to 6, and 8) or increased 0.10 unit (Experiments 7 and 9). The ion products calculated for the 15th day of equilibration were based on the pH of the solution on the 13th day, before the composition of the gas mixture was changed. A decrease in the negative logarithm of the ion product during this period of equilibration indicates that some calcium phosphate was redissolved; an increase indicates that it was reprecipitated.

The rate of precipitation of calcium phosphate varies with the amount

of solid calcium phosphate present in the solution. When no solid calcium phosphate was present, a solution which contained as much as 6.1 mg. of Ca and 3.5 mg. of P per 100 cc. (Experiment 1) formed no precipitate within the 15 days of the experiment, as shown by the constant value of the ion product. When a large amount of solid calcium phosphate was present, equilibrium was approached more rapidly from undersaturation (Experiments 7 and 9) than from supersaturation. Evidence that equilibrium was reached is adequate only in Experiments 7 to 9, in which nearly the same ion product constants were obtained at the end of the 13th day

TABLE I

Influence of Amount of Solid Phase on Solubility of Calcium Phosphate

The concentrations are expressed in mg. per 100 cc. of solution.

Experiment No	Initial composition*			Negative logarithm of ion product, $[Ca^{++}]^3 \times [PO_4^{\equiv}]^2$, after equilibration for				
	Dissolved		Solid calcium phosphate†					
	P	Ca		1 day	3 days	7 days	13 days	2 days after pH change‡
1	3.5	6.1	0	23.46	23.51	23.45	23.48	23.50
2	2.0	4.0	0	24.50	24.54	24.50	24.50	24.48
3	2.0	4.0	3	24.54	24.59	24.59	24.62	24.59
4	2.0	4.0	10	24.78	24.81	24.91	25.10	25.05
5	2.0	4.0	25	24.96	25.14	25.34	25.59	25.49
6	4.0	2.0	50	25.48	25.61	25.74	25.89	25.84
7	0.0	0.0	50	26.59	26.49	26.57	26.54	26.66
8	4.0	2.0	200	25.89	26.14	26.22	26.35	26.18
9	0.0	0.0	200	26.51	26.45	26.45	26.47	26.78

* In addition to these substances each solution contained 0.026 M $NaHCO_3$ and sufficient NaCl to bring the ionic strength to 0.158.

† The calcium phosphate precipitate was prepared about a month before from a solution which contained 25 mg. per cent of Ca, 12 mg. per cent of P, 0.72 gm. per cent of NaCl, and 0.26 gm. per cent of $NaHCO_3$.

‡ For interpretation of these values see the text.

when equilibrium was approached from both supersaturation and undersaturation, and in which the change in solubility product after the small change in pH indicated that a significant amount of calcium phosphate was either redissolved (Experiment 8) or reprecipitated (Experiments 7 and 9). In Experiments 4 to 6, both in this series and in several other series of similar experiments not recorded here, very small amounts of calcium phosphate, scarcely beyond the limits of error of analysis, were redissolved during the last 2 days when the solutions were equilibrated at a more acid pH. The re-solution of these small amounts of calcium

phosphate appears to indicate that equilibrium was also reached in these experiments. This is entirely possible, since equilibrium may be obtained at different solubility levels (see Table II). It is difficult to prove, because solution or precipitation proceeds so slowly when these small amounts of solid are present.

Effect of Concentration of Precipitating Solution and Age of Precipitate on Solubility of Calcium Phosphate (Table II)—Calcium phosphate precipitates were allowed to form for the indicated lengths of time from solutions of sufficient volume to give about 0.8 gm. of precipitate. Each precipitate was equilibrated first for a day with 40 cc. of bicarbonate-salt

TABLE II

Effect of Concentration of Precipitating Solution and Age of Precipitate on Solubility of Calcium Phosphate

The concentrations are expressed in mg. per 100 cc. of solution.

Experiment No.	Ppt. prepared from solution containing		Age of ppt.	Equilibrium approached							
				From supersaturation				From undersaturation			
	P	Ca		P	Ca	pH	pK'_{sp}	P	Ca	pH	pK'_{sp}
			days								
10	9	19	1	1.64	2.38	7.36	25.2	1.43	2.22	7.36	25.4
11	30	67	1	2.35	1.48	7.39	25.4	2.55	1.21	7.39	25.6
12	30	67	3	1.11	2.14	7.39	25.6	1.28	1.91	7.39	25.6
13	30	67	10	0.97	2.06	7.39	25.7	1.06	1.91	7.39	25.8
14	30	67	20	0.83	1.83	7.39	26.1	0.97	1.76	7.39	26.0
15	12	25	270*	1.03	1.08	7.38	26.6	0.92	1.31	7.39	26.4
16	6	13	9	1.03	2.48	7.41	25.4	1.10	2.38	7.44	25.4
17	9	22	9	0.72	2.77	7.41	25.6	0.79	2.52	7.44	25.6
18	9	14	9	2.36	1.21	7.40	25.6	2.00	1.38	7.42	25.5
19	20	47	9	0.60	2.97	7.42	25.6	0.62	2.58	7.44	25.7
20	20	41	9	1.45	1.60	7.41	25.7	1.45	1.52	7.43	25.7
21	40	94	9	0.51	2.72	7.46	25.8	0.54	2.87	7.44	25.7

* Kept for about 8 months in a stoppered flask.

solution which contained initially 4 mg. of Ca and 2 mg. of phosphorus per 100 cc. of solution (equilibrium approached from supersaturation), then for another day with 40 cc. of bicarbonate-salt solution which contained 2 mg. of Ca per 100 cc. of solution and no phosphate (equilibrium approached from undersaturation). Supernatant solutions and precipitates were separated from each other by means of a siphon and by centrifugation.

The solubility varies primarily in inverse relation to the age of the precipitate, extending from pK'_{sp} 25.2 to 26.6 (Experiments 10 to 15). The smaller solubility of the precipitates formed from the more concen-

trated solutions (Experiments 16 to 21) is probably attributable to more rapid formation of the precipitate. Solubility is essentially independent of the ratio of calcium to phosphorus in the supernatant solution (Experiments 17 to 20). In each experiment, the nearly identical solubility

TABLE III

Effects of Citrate, Magnesium, and Pyrophosphate on Solubility of Calcium Phosphate Precipitates

The concentrations are expressed as mg. of P, Ca, Mg, and citric acid per 100 cc. of solution.

Experiment No.	Ppt. prepared from solution containing					Age of ppt.	Equilibrium approached				
							From supersaturation				From undersaturation
	Orthophosphate	Ca	Mg	Citrate	Pyrophosphate		P	Ca	pH	pK'_{sp}	pK'_{sp}
						days					
22	9	20	0	0	0	1.5	1.21	2.72	7.38	25.2	25.5
23	9	20	0	6	0	1.5	1.67	3.48	7.35	24.7	24.7
24	9	20	0	14	0	1.5	2.00	3.80	7.32	24.5	24.4
25	9	20	2.5	0	0	1.5	1.67	3.02	7.35	24.9	25.1
26	9	20	5.0	0	0	1.5	3.03	6.62	7.40	23.2	23.5
27	9	20	2.5	6	0	1.5	4.94	2.44	7.32	24.6	24.6
28	9	19	2.5	7	0	1.5	4.12	6.34	7.39	23.1	24.3
29	9	19	2.5	7	0	1.5	4.20	8.82	7.39	22.6	23.7
30	9	19	2.5	7	0	1.5	5.17	3.02	7.39	23.8	24.6
31	9	19	2.5	7	0	1.5	2.82	3.08	7.38	24.3	24.7
32	8	17	2.5	7	0	1.5	4.50	2.88	7.35	24.1	24.4
33	8	19	2.5	7	0	1.5	4.70	2.51	7.32	24.3	24.6
34	9	19	0	0	0	13	1.22	1.51	7.32	26.1	26.1
35	9	19	5.0	0	0	13	1.23	3.38	7.34	25.1	25.1
36	11	23	7.5	0	0	13	1.50	4.47	7.31	24.6	24.6
37	15	32	5.0	10	0	13	1.52	3.20	7.41	24.8	24.8
38	15	33	5.0	10	1.0	13	1.74	3.34	7.41	24.6	24.4
39	15	36	5.0	10	3.0	13	3.07	9.99	7.45	22.6	22.6
40	15	39	5.0	10	5.0	13	3.25	9.95	7.45	22.5	22.5
41	15	32	5.0	10	0.5*	13	1.79	3.12	7.41	24.7	24.8

* Added as thiamine pyrophosphate.

values obtained when equilibrium was approached from supersaturation and from undersaturation show that the system was in equilibrium.

Effects of Citrate, Magnesium, and Pyrophosphate on Solubility of Calcium Phosphate Precipitates (*Table III*)—The procedure for the preparation of the precipitates and the determination of their solubility was essentially the same as has been described. Magnesium was added as magnesium

chloride to both the solutions from which calcium phosphate was precipitated and to the solutions in which the solubility was measured. Citric acid, added as sodium citrate, was rapidly decomposed during the period of precipitation. When precipitation proceeded for more than 2 days, the concentration was maintained at about 2.5 mg. per 100 cc., the amount present in blood (9), by the daily addition of about 3 mg. of sodium citrate per 100 cc. of solution. Citric acid was present initially in a concentration of 4 mg. per 100 cc. in the solutions used to test solubility; in Experiment 24 it was present in twice this concentration. During the periods of equilibration the citric acid concentrations decreased to less than 1 mg. per cent. The addition of metaphen in amounts sufficient to give a concentration of 1 part in 400,000, or 0.25 gm. of thymol, prevented the development of a turbidity on long equilibration, which was difficult to remove by filtration. Inorganic pyrophosphate was precipitated from the solutions along with orthophosphate and could not be detected in the supernatant solutions (less than 0.2 mg. of pyrophosphate phosphorus per 100 cc. of solution). It was not added as such to the solutions used to determine the solubility of the precipitates.

The solubility of calcium phosphate increases with the amount of citrate (Experiments 22 to 24) and with the amount of magnesium (Experiments 25 and 26, and 34 to 36) in the equilibrium mixture. When both magnesium and citrate are present, the effects are additive and the amounts of dissolved calcium and phosphate correspond roughly to the amounts present in the ultrafiltrate of blood plasma (Experiments 27 to 33). This effect of magnesium and citrate on solubility was not as pronounced when the precipitates were equilibrated 13 days instead of 1.5 days. Inorganic pyrophosphate has no very pronounced effect until it is present in an amount equal to about one-fifth of the orthophosphate (Experiments 37 to 40). Thiamine pyrophosphate has no effect when present in an amount much larger than in the blood plasma (Experiment 41). When present in an amount equivalent to 1.0 mg. of phosphorus per 100 cc., it greatly increases the solubility of calcium phosphate (not shown in Table III).

DISCUSSION

The changes in solubility of calcium phosphate with age of the precipitate parallel changes in composition of the precipitate previously demonstrated (8), and can reasonably be attributed to these changes. The effects of magnesium and citrate are larger than would be expected on the basis of their formation of slightly dissociated magnesium phosphate (10) and calcium citrate complexes (6) respectively. Both citrate and magnesium probably affect solubility by inclusion in and alteration of the structure of

the precipitate. Magnesium appears to decrease the rate at which the composition of the precipitate is changed, after it is first formed from solutions of high concentrations of calcium and phosphate (8). The solvent action of magnesium on these precipitates furnishes a theoretical basis for reports that magnesium decreases the incidence of kidney stones (11), that high salivary magnesium is associated with dental caries (12), and that serum magnesium rises in alkalosis (13).

The pK'_{sp} of tricalcium phosphate calculated on the basis of the amounts of calcium and phosphate found in serum ultrafiltrate, 5.4 and 4.0 mg. per 100 cc. respectively (1), and a pH of 7.38, is 23.3. When calculated on the basis of ionic calcium and inorganic non-creatine phosphate, about 4.8 and 1.5 mg. respectively (14, 7), the pK'_{sp} is 24.3. The average value for precipitates formed in the presence of citrate and magnesium, and about 3 days old, is 24.1 (Experiments 27 to 33). Precipitates of this age have about the same composition as dentin or bone (8). It is possible that younger precipitates have a still higher solubility, but this is probably limited by the solubility of dicalcium phosphate.

The marked effects of pyrophosphate are observed when pyrophosphate is present in the precipitate but cannot be detected in the supernatant solution. Inorganic pyrophosphatase is present in high concentration in blood cells and muscle tissues (15, 16) and inorganic pyrophosphate has been reported to be present in muscle after exercise (17). If high levels of calcium and phosphate in blood serum are maintained by inorganic pyrophosphate, calcium pyrophosphate should be present when calcified tissue is in contact with tissue fluid. In experiments not recorded in this paper it was determined that the bone substance dissolved by a short exposure of fresh bone to hydrochloric acid solution did not contain measurable amounts of pyrophosphate.

SUMMARY

Calcium phosphate is precipitated from solutions of the serum salts when calcium and phosphate are present in excess of the amounts found in serum ultrafiltrate. When precipitation has once begun, it continues until the amounts of calcium and phosphate in the supernatant solution are much less than the amounts present in serum ultrafiltrate.

The calcium phosphate which is precipitated from serum salt solutions has a varying solubility. It is most soluble when first formed from dilute solutions; the solubility decreases as the precipitate remains in the solution. Reliable solubility measurements require the presence of large amounts of solid in the equilibrium mixtures. Solubility values were established by approach from both supersaturation and undersaturation. Changes in solubility may be attributed to changes in the composition of the precipitate.

The presence of magnesium and citrate in the precipitate and in the supernatant solution increases the solubility of calcium phosphate precipitate. When these substances are present in the supernatant solution in amounts equivalent to their concentrations in serum, the solubility of the precipitate measured a few days after its formation corresponds closely to the amounts of ionic, and approaches values of diffusible calcium and phosphate in blood serum.

The presence of pyrophosphate in the precipitating solution increases the solubility of calcium orthophosphate. Inorganic pyrophosphate is precipitated and cannot be detected in the supernatant solution.

The author is indebted in Dr. D. F. Robertson of Merck and Company, Inc., for a supply of thiamine pyrophosphate.

BIBLIOGRAPHY

1. Schmidt, C. L. A., and Greenberg, D. M., *Physiol. Rev.*, **15**, 297 (1935).
2. Greenwald, I., *J. Biol. Chem.*, **143**, 711 (1942).
3. Logan, M. A., and Taylor, H. L., *J. Biol. Chem.*, **119**, 293 (1937).
4. Sendroy, J., Jr., and Hastings, A. B., *J. Biol. Chem.*, **71**, 783, 797 (1926–27).
5. Holt, L. E., Jr., La Mer, V. K., and Chown, H. B., *J. Biol. Chem.*, **64**, 509, 567 (1925).
6. McLean, F. C., and Hastings, A. B., *J. Biol. Chem.*, **108**, 285 (1935).
7. Göbell, O., and Janda, K., *Z. Kinderh.*, **63**, 524 (1942); *Chem. Abstr.*, **37**, 5770 (1943).
8. Kuyper, A. C., *J. Biol. Chem.*, **159**, 411 (1945).
9. Kuyper, A. C., and Mattill, H. A., *J. Biol. Chem.*, **103**, 51 (1933).
10. Greenwald, I., Redish, J., and Kibrick, A. C., *J. Biol. Chem.*, **135**, 65 (1940).
11. Cunningham, I. J., and Cunningham, M. M., *New Zealand J. Sc. and Tech.*, **19**, 529 (1938).
12. Krasnow, F., and Obatt, E. B., *J. Dent. Res.*, **19**, 305 (1940).
13. Wolff, R., Rangier, M., and Bourguard, A., *Compt. rend. Soc. biol.*, **122**, 391 (1936).
14. Morison, R. S., McLean, R., and Jackson, E. B., *J. Biol. Chem.*, **122**, 439 (1937–38).
15. Sjöberg, K., *Acta physiol. Scand.*, **1**, 220 (1940).
16. Itakawa, T., *J. Biochem.*, Japan, **33**, 249 (1941).
17. Ferdman, D. L., and Feĭnshmidt, O. I., *Biokhimiya*, **2**, 776 (1937); *Chem. Abstr.*, **32**, 2190 (1938).

THE EXCRETION OF "FOLIC ACID" THROUGH THE SKIN AND IN THE URINE OF NORMAL INDIVIDUALS*

By B. CONNOR JOHNSON, T. S. HAMILTON, and H. H. MITCHELL

(*From the Division of Animal Nutrition, University of Illinois, Urbana*)

(Received for publication, April 13, 1945)

A preliminary study by Wright *et al.* (1) with the *Streptococcus lactis* R procedure (2) indicated that human urine contains only small amounts of "folic acid." They reported 0.0047 to 0.0064 γ (2) per cc. In a more complete study Wright and Welch (3) reported the folic acid analyses of forty-two samples of urine from fifteen individuals with the *Lactobacillus casei* procedure (4). They reported 0.0038 to 0.0238 mg. (average 0.0108) excreted per day and stated, "Evidently folic acid is unlike other members of the vitamin B complex in that only a trace appears in the urine of man (<1 per cent. of the probable dietary intake)."

In our work on the effect of temperature on the excretion of various nutrients through the skin and in the urine, we have found the dermal excretion of thiamine, pyridoxine riboflavin, nicotinic acid, pantothenic acid, biotin, and choline to be practically negligible as compared with urinary excretion. However, folic acid has proved to be an interesting exception, in that more has been found to be excreted in the sweat under hot environmental conditions than in the urine.

EXPERIMENTAL

Four adult male subjects, age 21 to 28 years, were maintained for 8 hours per day under hot moist conditions (37.7°, 70 per cent relative humidity) for two 5 day periods. The subjects were kept on a constant diet throughout the experiment. During the second experimental period the diet was supplemented with 5.3 gm. of brewers' yeast per day, representing 5.5 γ of added free folic acid (5), or 1.06 mg. of total folic acid (6).

Daily (24 hours) urinary collections were made throughout the 5 day periods and composite samples for each subject were analyzed for folic acid. Complete 8 hour sweat collections were made each day and the samples combined as in the case of urine. All samples were preserved with acetic acid and stored in the refrigerator.

The urine and the samples of sweat plus body washings were analyzed for folic acid with the *Streptococcus lactis* procedure of Luckey, Briggs,

* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Illinois.

and Elvehjem (7) and the *Lactobacillus casei* procedure of Landy and Dicken (4). Two standards were used for these assays; one was a folic acid concentrate kindly supplied by Dr. R. J. Williams of the University of Texas and the other was a solution of the "*Lactobacillus casei* factor" kindly supplied by Dr. E. L. R. Stokstad of the Lederle Laboratories, Inc.

The daily excretions of folic acid in the urine (24 hours) and in the sweat (8 hours), as determined by both organisms, are reported in Table I in terms of Williams' folic acid concentrate (micrograms (2)). The ratios of the hourly dermal to the hourly urinary losses are also given in Table I.

On the day following each experimental period, undiluted sweat was

TABLE I

Daily Excretions of Folic Acid in Urine and in Sweat

Period No.	Subject	*Lactobacillus casei* assay			*Streptococcus lactis* assay		
		Dermal loss, 8 hrs.	Urinary loss, 24 hrs.	Ratio, dermal to urinary excretion of folic acid per hr.	Dermal loss, 8 hrs.	Urinary loss, 24 hrs.	Ratio, dermal to urinary excretion of folic acid per hr.
		γ	γ		γ	γ	
I. No dosage	C	45	15	9.0	20	7	8.6
	D	38	52	2.2	22	12	5.5
	E	26	15	5.2	20	7	8.6
	F	27	11	7.4	15	6	7.5
Average.		34	23	5.95	19	8	7.5
II. Yeast dosage	C	29	20	4.35	18	7	7.7
	D	38	58	2.0	17	14	3.6
	E	12	20	1.8	9	8	3.4
	F	28	12	7.0	14	6.5	6.5
Average.		27	27	3.8	14	9	5.3
" (both periods).		30	25	4.9	17	8	6.4

collected under the same hot moist environmental conditions. These samples were preserved and analyzed as before. The folic acid concentration of these sweat samples is given in Table II.

When *Lactobacillus casei* factor (Stokstad) was used as a reference standard in place of folic acid (Williams), the values obtained were 1.8 (1.6 to 2.1) times as high. This increase in values is due to the fact that *L. casei* factor was slightly more than half as active as was folic acid for the growth of *L. casei*. The curve for the *L. casei* factor with both *L. casei* and *Streptococcus lactis* was markedly sigmoid, little increase in growth being observed until a threshold level was present. The values

obtained with the *L. casei* factor (Stokstad) standard with *S. lactis* as the assay organism were approximately 15 times as high as those with the folic acid (Williams) standard. This relative inactivity of *L. casei* factor for *S. lactis* has been reported several times (8).

DISCUSSION

The most interesting aspect of the results appears to be the relatively large excretion of folic acid through the skin, as compared to the urinary excretion under profuse sweating conditions. As shown in Table I, the average ratio of skin excretion to urinary excretion is 4.9 with *Lactobacillus casei* and 6.4 with *Streptococcus lactis*. This ratio for some of the other B

TABLE II

Folic Acid Concentration of Undiluted Sweat

Period No.	Subject	*Lactobacillus casei* assay	*Streptococcus lactis* assay
		γ per cc.	*γ per cc.*
I	C	0.0089	0.0062
	D	0.031	0.013
	E	0.0062	0.0053
	F	0.0046	0.0023
Average		0.0127	0.0067
II	C	0.0024	0.0027
	D	0.0095	0.0051
	E	0.0018	0.0042
	F	0.0058	0.0033
Average		0.0049	0.0038
" (both periods)		0.0088	0.0053

vitamins is by comparison[1] 0.48 for nicotinic acid, 0.24 for pantothenic acid, and 0.5 for pyridoxine; that is, the ratio is one-tenth to one-twentieth that for folic acid.

However, as reported by Wright *et al.* (1, 3), we also have found that human urine contains only very small amounts of folic acid. Thus if the human requirement for folic acid is 0.5 to 1.0 mg. units, as suggested by Williams (9), even the total *Streptococcus lactis*-active folic acid excretion for 24 hours under sweating conditions (approximately 0.06 mg. unit per 24 hours for the sweat and urine) apparently would not affect materially the folic acid requirement.

Since this work was completed, several reports (6, 10–12) have appeared

[1] Unpublished data.

showing that many sources of folic acid contain a conjugate which can be hydrolyzed by the enzyme "vitamin B_c conjugase" (11, 12). It seemed possible in view of these findings that the low urinary folic acid excretion might be due to the excretion of folic acid in urine largely as a conjugate.

This postulate has been tested by the action of a vitamin B_c conjugase preparation prepared from hog kidney by the method of Bird *et al.* (11) on pooled urine samples, as well as on yeast extract (Difco) which was used to test the activity of the enzyme preparation. The values obtained are given in Table III.

From Table III it is evident that there is no folic acid conjugate, such as that present in yeast, being excreted in urine. We have also found, as reported by Wright and Welch (3), that the folic acid content of urine is not increased by digestion with taka-diastase.

TABLE III

Effect of Enzyme Digestion on Folic Acid Content of Hog Urine

Sample	Enzyme treatment	Folic acid content
		γ per gm.
Yeast extract	None	13
" "	Treated	60
Pooled Urine 2	None	0.010
" " 2	Treated	0.009
" " 1	None	0.010
" " 1	Treated	0.0092

SUMMARY

1. It has been shown that the human excretes folic acid in the sweat as well as in the urine.

2. Approximately 5 times as much active folic acid as determined by *Lactobacillus casei* was excreted per hour in the sweat as in the urine, and about 6 times as much as determined by the *Streptococcus lactis* assay under conditions of profuse sweating.

3. The amount of folic acid found in urine was not increased by incubation with vitamin B_c conjugase.

BIBLIOGRAPHY

1. Wright, L. D., McMahan, J. R., Cheldelin, V. H., Taylor, A., Snell, E. E., and Williams, R. J., *Univ. Texas Pub., No. 4137*, 38 (1941).
2. Mitchell, H. K., and Snell, E. E., *Univ. Texas Pub., No. 4137*, 36 (1941).
3. Wright, L. D., and Welch, A. D., *Science*, **98**, 179 (1943).
4. Landy, M., and Dicken, D. M., *J. Lab. and Clin. Med.*, **27**, 1086 (1942).
5. Woods, A. M., Taylor, J., Hofer, M. J., Johnson, G. A., Lane, R. L., and McMahan, J. R., *Univ. Texas Pub., No. 4237*, 84 (1942).

6. Mims, V., Totter, J. R., and Day, P. L., *J. Biol. Chem.*, **155**, 401 (1944).
7. Luckey, T. D., Briggs, G. M., Jr., and Elvehjem, C. A., *J. Biol. Chem.*, **152**, 157 (1944).
8. Luckey, T. D., Teply, L. J., and Elvehjem, C. A., *Science*, **100**, 201 (1944).
9. Williams, R. J., *J. Am. Med. Assn.*, **119**, 1 (1942).
10. Binkley, S. B., Bird, O. D., Bloom, E. S., Brown, R. A., Calkins, D. G., Campbell, C. J., Emmett, A. D., and Pfiffner, J. J., *Science*, **100**, 36 (1944).
11. Bird, O. D., Binkley, S. B., Bloom, E. S., Emmett, A. D., and Pfiffner, J. J., *J. Biol. Chem.*, **157**, 413 (1945).
12. Laskowski, M., Mims, V., and Day, P. L., *J. Biol. Chem.* **157**, 731 (1945).

SOME AMINO ACID ANALYSES OF HEMOGLOBIN AND β-LACTOGLOBULIN

By G. L. FOSTER

(*From the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York*)

(Received for publication, April 18, 1945)

Quantitative determinations of aspartic and glutamic acids, leucine, glycine, lysine, arginine, phenylalanine, and tyrosine have been made by the isotope dilution method (1) on acid hydrolysates of horse hemoglobin. Two specimens of β-lactoglobulin have been similarly examined for the first five of the amino acids mentioned. A summary of the results of the analyses is given in Table I, together with some analyses from the literature for comparison. The data and experimental observations from which the results in Table I were calculated are given in Tables II and III.

EXPERIMENTAL

Materials

The standard isotopic amino acids were synthesized in this laboratory and were of satisfactory purity as evinced by analysis for C, H, and N; also for Cl in the cases in which the amino acid was used as its hydrochloride (lysine, arginine, and glutamic acid) and by specific rotation in the case of the $l(+)$-glutamic acid.

The horse carboxyhemoglobin was prepared from sedimented and washed cells by electrodialysis. The crystalline material was twice recrystallized. It appears homogeneous in electrophoresis. The dry protein had 0.334 per cent Fe and 16.8 per cent N. For the analyses the air-dried protein was weighed and corrected for moisture (5.6 per cent).

Sample A of the β-lactoglobulin was a twice crystallized preparation kindly given us by Professor R. K. Cannan. It was received as a moist paste. The amount available for analysis was 7.05 gm. of protein, as determined by Kjeldahl analysis, 15.6 per cent being taken as the nitrogen content of this protein (9). Sample B was part of a preparation presented to Dr. E. Brand of this Department by Dr. G. Haugaard to whom we convey our thanks. It was weighed as an air-dried powder having 6.3 per cent moisture.

Procedure

The essential operations in the isotope dilution method as used in this work are (1) hydrolysis of a weighed sample of protein for 18 to 20 hours

with 15 to 20 times its weight of boiling 6 N HCl, (2) addition to the hydrolysate of a weighed amount of isotopic *dl*-amino acid whose N^{15} excess is known, (3) isolation from the hydrolysate of a specimen of the natural (*l*) isomer of the amino acid in a high state of purity, (4) determination of N^{15} excess in the isolated compound by the mass spectrograph.

In practice several different amino acids may be determined in the same hydrolysate.

The addition of *dl* isotopic compounds to the hydrolysate necessitates the isolation of a specimen of the pure *l* isomer from the solution which

TABLE I

Amino Acids of Horse Hemoglobin and β-Lactoglobulin

Amino acid	Hemoglobin				β-Lactoglobulin				
	This work		Value from literature	Bibliographic reference	This work			Value from literature	Bibliographic reference
						Sample A	Sample B		
	*residues per mole**	*per cent*	*per cent*		*residues per mole**	*per cent*	*per cent*	*per cent*	
Glutamic	39	8.5	6.8	(2)	55	19.1	19.0	21.5	(3)
								19.0	†
								18.7	(4)
Aspartic	52	10.3	9.3	(2)	36	11.3	11.2	9.9	(3)
								11.0	†
Leucine	77	15.1	15.7	(5)	50	15.6	15.7	15.4	(5)
Glycine	50	5.6			8	1.5	1.5		
Lysine	39	8.6	8.1	(6)	33	11.4	11.4	9.8	(3)
Arginine	14	3.7	3.7	(3, 7)					
Phenylalanine	27	6.8							
Tyrosine	11	3.0	3.2	(8)					

* The molecular weights of hemoglobin and β-lactoglobulin are taken to be 66,700 and 42,000, respectively.

† Snell, E. E., personal communication.

contains both *l* and *d*. In some cases the racemic compound is less soluble than the desired *l* component and cannot be removed by recrystallizations. For each different amino acid sought, some preliminary work must therefore be done to find a way to isolate a specimen of the *l* isomer. Because of this and also because it is essential that the compounds brought to isotope analysis be in a high state of purity (or at least free from other nitrogen compounds), it seems necessary to outline briefly the procedure for the isolation of each amino acid.

The purification of each compound isolated from the hydrolysate was effected by recrystallizations under conditions known to lead to the pure

l isomer. Two or more recrystallizations were made *after* the correct specific rotation was attained. The purity of the compounds was established by (1) constancy of isotope concentration at successive stages of

TABLE II

Analyses of Horse Hemoglobin

Experiment No.	Amino acid added			N^{15} excess in compound isolated at successive stages of recrystallization		Weight of protein hydrolyzed (*B*)	Amino acid in protein* $\left(\frac{C_0}{C} - 1\right) \times \frac{100A}{B}$
	Compound	Amount of *l*-amino acid (*A*)	N^{15} excess (C_0)	(*C*)	Mean (*C*) values		
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
		gm.	*atom per cent*	*atom per cent*	*atom per cent*	*gm.*	*per cent*
4	Tyrosine	0.0740	6.85	0.694, 0.693, 0.710	0.699	21.65	3.0
5	"	0.0734	6.85	0.602, 0.602	0.602	25.60	3.0
7	"	0.0768	6.85	0.645, 0.654, 0.647	0.649	24.58	3.0
4	Phenylalanine	0.0739	6.79	0.324, 0.320, 0.327	0.324	21.65	6.8
5	"	0.1296	6.79	0.466, 0.478, 0.472	0.472	25.60	6.8
7	"	0.1522	6.79	0.552, 0.563, 0.563	0.559	24.58	6.9
1	Arginine	0.0850	8.72	0.845, 0.831	0.838	21.60	3.7
4	"	0.0710	8.72	0.722, 0.697	0.720	21.65	3.7
5	"	0.0614	8.72	0.530, 0.527, 0.529	0.529	25.60	3.7
7	Lysine	0.1372	9.09	0.559, 0.557	0.558	24.58	8.5
8	"	0.1542	9.09	0.674, 0.667, 0.671	0.671	22.13	8.7
11	"	0.1430	9.09	0.690, 0.691, 0.691	0.691	20.39	8.5
5	Glutamic acid	0.2030	4.52	0.383, 0.384, 0.381	0.383	25.60	8.6
7	" "	0.2211	4.52	0.437, 0.440, 0.435	0.437	24.58	8.4
8	" "	0.2446	4.52	0.528, 0.528, 0.528	0.528	22.13	8.4
8	Aspartic "	0.1642	6.75	0.452, 0.449, 0.450	0.450	22.13	10.4
11	" "	0.1420	6.75	0.421, 0.426	0.424	20.39	10.4
8	Glycine	1.1869	1.15	0.570, 0.574, 0.565	0.570	22.13	5.5
11	"	0.3498	3.38	0.791, 0.789, 0.787	0.789	20.39	5.6
2	Leucine	0.0767	6.73	0.646, 0.642, 0.626	0.638	4.85	15.1
3	"	0.0621	6.73	0.547, 0.552, 0.537	0.545	4.69	15.0
6	"	0.0300	6.73	0.507, 0.508	0.508	2.44	15.1
10†	"	0.0210	6.73	0.525	0.525	1.65	15.0

* The values in Column 8 are calculated from the mean values of (*C*) which are shown in Column 6.

† Hydrolysis continued for 94 hours.

recrystallization, (2) melting point in the cases of benzoylglycine, dibenzoyllysine, and the benzenesulfonyl derivatives of leucine and phenylalanine, and (3) nitrogen content by Kjeldahl analysis.

Isolation of Amino Acids—After the isotopic amino acids have been

added to the hydrolysate, the solution is concentrated to remove excess HCl, taken up in water, and treated with a suspension of Cu_2O, as recommended by Bailey *et al.* (2) to remove humin and cystine. The filtrate, after the removal of copper, is adjusted to pH 5 (approximate) and concentrated for the crystallization of tyrosine.

Tyrosine—The crude tyrosine is a mixture of *l* and *dl* isomers which does not separate satisfactorily on recrystallization. It is converted to the copper salt by being boiled with copper carbonate and the filtrate is stored in an ice bath for crystallization of the *l* salt. The solubilities in water

TABLE III

Analyses of β-Lactoglobulin

Lactoglobulin specimen	Amino acid added			N^{15} excess in compound isolated at successive stages of recrystallization		Weight of protein hydrolyzed (B)	Amino acid in protein* $\left(\frac{C_0}{C} - 1\right) \times \frac{100A}{B}$
	Compound	Amount of *l*-amino acid (A)	N^{15} excess (C_0)	(C)	Mean (C) values		
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
		gm.	*atom per cent*	*atom per cent*	*atom per cent*	*gm*	*per cent*
A	Glutamic acid	0.1484	1.52	0.448, 0.448	0.448	7.05	19.1
B	" "	0.1413	4.52	0.470, 0.476	0.473	6.37	19.0
A	Aspartic "	0.0681	6.75	0.538, 0.532, 0.532	0.534	7.05	11.3
B	" "	0.0624	11.82	0.947, 0.961	0.954	6.37	11.2
A	Lysine	0.0660	9.09	0.689, 0.683, 0.687	0.686	7.05	11.4
B	"	0.0641	9.09	0.736, 0.736	0.736	6.37	11.4
A	Leucine	0.0688	6.73	0.392, 0.393, 0.403	0.396	7.05	15.6
B	"	0.0698	6.73	0.436, 0.441	0.439	6.37	15.7
A	Glycine	0.3147	1.99	1.474, 1.496	1.485	7.05	1.5
B	"	0.2365	2.42	1.73, 1.73	1.73	6.37	1.5

* The values in Column 8 are calculated from the mean values of (C) which are shown in Column 6.

at 0° of *dl*- and *l*-tyrosine copper salts were found to be respectively 46 and 21 mg., calculated as tyrosine per 100 cc. of solution. From the weight and the rotation of the crude tyrosine and the solubility data one can calculate the volume required to keep all of the *dl*-tyrosine copper salt in solution while a specimen of the *l* salt crystallizes. The *l*-tyrosine copper salt is suspended in a small volume of water and treated with an equivalent amount of HCl. The tyrosine so obtained should have the correct rotation (10). It is recrystallized two or three times, a sample at each stage being saved for N^{15} analysis.

Leucine—The filtrate from the crude tyrosine is adjusted to about pH

6.0, concentrated to smaller volume, and left several days in the cold. From the "crude leucine" fraction, *l*-leucine is isolated as its salt with 2-bromotoluene-5-sulfonic acid (11), the solubilities of the *l* and *dl* salts in 1 N HCl at 0° being about 1.2 and 4.1 gm. respectively of the salt per 100 cc. of solution. The leucine is purified as the benzenesulfonyl derivative which has very favorable properties; *e.g.*, m.p. 119° (corrected), $[\alpha]_D^{22} = -53°$ (in the presence of 6 moles of NaOH and $c = 2$), solubility in water at 0°, *l* form 47 mg., *dl* form 118 mg. per 100 cc. The leucine salt of bromotoluenesulfonic acid is benzenesulfonated directly.

Phenylalanine—If the protein is not too poor in phenylalanine, this amino acid will be present in the "crude leucine" fraction and will pass into the filtrate from the leucine salt, from which it may be isolated as its salt with 2,5-dibromobenzenesulfonic acid (11). Final purification of the *l*-phenylalanine is effected by recrystallization of its benzenesulfonyl derivative from hot water; m.p. 133° (corrected), $[\alpha]_D^{22} = -35.5°$ (in the presence of 6 moles of NaOH and $c = 2$), solubilities in water at 0°, *l* form 37 mg., and *dl* form 85 mg. of the sulfonyl derivative per 100 cc. of solution.

Arginine—From the filtrate from the "crude leucine" fraction arginine is isolated as the monoflavianate in the usual way. The monoflavianate is recrystallized from 0.1 N HCl with a volume sufficient to keep in solution the amount of *dl*-arginine that was added to the hydrolysate. The solubilities of *l*- and *dl*-arginine monoflavianates in 0.1 N HCl at 25° were found to be 29 and 56 mg. respectively (as arginine flavianate) per 100 cc. The *l*-arginine monoflavianate is converted to the di-3,4-dichlorobenzenesulfonate (11) by dissolving the monoflavianate in hot 1 N HCl and adding a large excess of the dichlorobenzenesulfonic acid. This salt is recrystallized several times. The anhydrous salt has $[\alpha]_D^{23} = +8.0°$ ($c = 6$ in 95 per cent alcohol); the solubilities of the *l*- and the *dl*-arginine salts in 1 N HCl at 0° were respectively 117 and 112 mg. of salt per 100 gm. of solution.

Lysine—The filtrate from arginine flavianate is made strongly acid with HCl and shaken with butanol to remove the excess flavianic acid. The excess of HCl is removed by distillation and histidine is precipitated in the usual way with $HgCl_2$ at pH 6.8 (12). After the removal of mercury with H_2S, lysine is precipitated with phosphotungstic acid. Lysine is recovered from the phosphotungstate as the monohydrochloride which is converted to the dibenzoyl derivative and recrystallized from acetone until it has $[\alpha]_D^{22} = -8.6°$ ($c = 5$ in methyl alcohol) and a melting point of 149.5–150° (corrected). At this stage a sample is saved for analysis and the rest is recrystallized twice, a sample being taken for isotope analysis at each stage. Undoubtedly a more convenient method for preparing specimens of *l*-lysine of proved purity can be found.[1]

[1] The procedure described by Shemin in the following paper (13) is much better.

Glutamic Acid—The filtrate from lysine phosphotungstate is freed from phosphotungstic acid by extraction with butanol-ether mixture. The dicarboxylic acid fraction is obtained in the usual way with barium hydroxide and alcohol. From this fraction glutamic acid is isolated and purified as the hydrochloride (1).

Aspartic Acid—The filtrate from glutamic hydrochloride, after appropriate treatment, yields copper aspartate which is purified as the free acid by recrystallization from water until it has $[\alpha]_D^{22} = +25.5°$ ($c = 2$ in 1 N HCl). The substance is then recrystallized two or three times more, a sample being saved at each stage for analysis.

Glycine—The filtrate from the barium dicarboxylates is freed from alcohol and barium and treated with potassium trioxalatochromiate according to Bergmann and Fox (14) for the isolation of glycine. The glycine is purified as the benzoyl derivative; m.p. 190° (corrected).

DISCUSSION

The figures in Table I refer to *only the l form* of the amino acid in the hydrolysate. Just how closely this coincides with the amount of amino acid in the original protein may be somewhat uncertain, for we do not know for every amino acid whether or not there is partial destruction or racemization during hydrolysis. The fact that most of the amino acids are stable, and are only very slowly racemized when boiled with acids, does not preclude the possibility that the compounds may be more labile while in peptide linkage. This possibility has been raised recently by Martin and Synge (15) to account for their finding of racemized acetylphenylalanine from a wool hydrolysate. They cite from the literature several instances wherein partly racemized phenylalanine has been isolated from protein hydrolysates. However, subsequent work by Gordon, Martin, and Synge (16) has thrown doubt on the validity of the finding of racemized acetylphenylalanine.

It has previously been shown (17) by the isotope dilution method that, in the hydrolysis of placenta and of tumor tissue, there is no detectable racemization of glutamic acid, at least 99 per cent of this amino acid in the hydrolysate being in the *l* form. A similar experiment regarding the phenylalanine in a hydrolysate of horse hemoglobin is now reported.

To the acid hydrolysate of 31.4 gm. of once crystallized horse hemoglobin[2] 0.800 gm. of *dl*-phenylalanine having 1.71 per cent N^{15} excess was added. The *l*-phenylalanine isolated had 0.264 per cent N^{15}, while the *dl*-phenylalanine isolated had 0.904 per cent N^{15}. From these data we calculate (*cf.* (17)) that the original hydrolysate contained 2.16 gm. of *l*-phenylala-

[2] This hemoglobin was a different lot and presumably less pure than that used for the other analyses.

nine and 0.04 gm. of *d*-phenylalanine derived from the protein. Thus, only 1.8 per cent of the total phenylalanine from the protein was the *d* isomer. This is about the magnitude of the error to be expected in the experiment and is not significant.

That there is destruction of phenylalanine in boiling 6 N HCl is indicated by the finding of ammonia and the odor of phenylacetaldehyde in the reaction mixture. An experiment designed to measure by isotope dilution the extent of this decomposition of phenylalanine in boiling 6 N HCl solution indicated a destruction of about 5.5 per cent of the compound in 40 hours. Hence, it seems likely that the figure for phenylalanine in Table I is 2 or 3 per cent too low (*i.e.* 6.8 per cent perhaps should be 7.0 per cent).

Leucine, on the other hand, seems to be more stable. In Experiment 10 (see Table II), hydrolysis was continued for 94 hours with no decrease in *l*-leucine found.

The values reported in Table I for glycine are subject to greater error than are the others because of the unfavorable ratios of $C_0:C$ in these experiments. These low ratios were the result of adding to the hydrolysates not only the nitrogen-labeled glycine but also weighed amounts of normal glycine in order to insure sufficient of the amino acid in the hydrolysate for isolation and purification. This was not done in the other analyses.

Comparison with other values in the literature is found in Table I. In general it seems, as would be expected, that the isolation methods (2, 3, 6) yield lower results. Two exceptions appear in Table I; namely, the higher glutamic acid figure for β-lactoglobulin by isolation (3) and the very close agreement of the arginine values for hemoglobin by isotope dilution and by Vickery's diflavianate method (3, 7). Our results agree well with those by the microbiological methods of Brand (5) for leucine, of Snell[3] for glutamic and aspartic acids, and of Lewis and Olcott (4) for glutamic acid.

The writer thanks Mr. William Saschek for the carbon and hydrogen analyses, and Mr. I. Sucher for the N^{15} determinations.

SUMMARY

Quantitative determinations of aspartic and glutamic acids, leucine, glycine, lysine, arginine, phenylalanine, and tyrosine have been made by the isotope dilution method on acid hydrolysates of horse hemoglobin. β-Lactoglobulin has been similarly examined for the first five of the amino acids mentioned. The results are compared with recent values in the literature. Evidence is presented which indicates that phenylalanine is slowly destroyed by boiling 6 N HCl but is not appreciably racemized.

[3] Snell, E. E., personal communication.

BIBLIOGRAPHY

1. Rittenberg, D., and Foster, G. L., *J. Biol. Chem.*, **133**, 737 (1940).
2. Bailey, K., Chibnall, A. C., Rees, M. W., and Williams, E. F., *Biochem. J.*, **37**, 360 (1943).
3. Chibnall, A. C., Rees, M. W., and Williams, E. F., *Biochem. J.*, **37**, 372 (1943).
4. Lewis, J. C., and Olcott, H. S., *J. Biol. Chem.*, **157**, 265 (1945).
5. Ryan, F. J., and Brand, E., *J. Biol. Chem.*, **154**, 161 (1944).
6. Vickery, H. B., and Leavenworth, C. S., *J. Biol. Chem.*, **79**, 377 (1928).
7. Vickery, H. B., *J. Biol. Chem.*, **132**, 325 (1940).
8. Folin, O., and Marenzi, A. D., *J. Biol. Chem.*, **83**, 89 (1929).
9. Chibnall, A. C., Rees, M. W., and Williams, E. F., *Biochem. J.*, **37**, 354 (1943).
10. Stein, W. H., Moore, S., and Bergmann, M., *J. Am. Chem. Soc.*, **64**, 724 (1942).
11. Doherty, D. G., Stein, W. H., and Bergmann, M., *J. Biol. Chem.*, **135**, 487 (1940).
12. Foster, G. L., and Shemin, D., in Organic syntheses, New York, **18**, 43 (1938).
13. Shemin, D., *J. Biol. Chem.*, **159**, 439 (1945).
14. Bergmann, M., and Fox, S. W., *J. Biol. Chem.*, **109**, 317 (1935).
15. Martin, A. J. P., and Synge, R. L. M., *Biochem. J.*, **35**, 91 (1941).
16. Gordon, A. H., Martin, A. J. P., and Synge, R. L. M., *Biochem. J.*, **37**, 79 (1943).
17. Graff, S., Rittenberg, D., and Foster, G. L., *J. Biol. Chem.*, **133**, 745 (1940).

AMINO ACID DETERMINATIONS ON CRYSTALLINE BOVINE AND HUMAN SERUM ALBUMIN BY THE ISOTOPE DILUTION METHOD*

By DAVID SHEMIN

(*From the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York*)

(Received for publication, April 18, 1945)

In this paper there is reported the determination, by the isotope dilution method (1), of glutamic acid, aspartic acid, tyrosine, and glycine in both bovine and human serum albumin, and of lysine in bovine serum albumin. A summary of the analytical results is given in Table I and the data for the individual experiments are given in Tables II and III.

The isotope dilution technique was also applied to the problem of the conversion of serine into glycine under conditions existing during the acid hydrolysis of proteins. It was found that although serine is partially destroyed (3) it is not converted to glycine.

EXPERIMENTAL

The preparation and analytical data of the standard isotopic amino acids (4) and the isolation and purification of the *l*-amino acids from the protein hydrolysates (5, 6) were essentially the same as those already described in reports from this laboratory. Weighed samples of the proteins, corrected for moisture, were hydrolyzed for 15 hours with 20 times their weight of 20 per cent HCl and known amounts of the different isotopic *dl*-amino acids were then added, after which the solution was boiled for 1 hour. Amino acids isolated from the hydrolysates were recrystallized under conditions known to lead to the pure *l* isomers (5, 6). Their purity was established by the nitrogen content, the constancy of isotope concentration at successive stages of recrystallization, the specific rotation, and, in the case of glycine, by the melting point of the *p*-toluenesulfonyl derivative.

Tyrosine—The hydrolysate containing the added amino acids was evaporated to dryness *in vacuo* and the residue dissolved in water and treated with cuprous oxide to remove cystine, as recommended by Bailey, Chibnall, Rees, and Williams (7). The filtrate, after being freed of copper

* The present analytical studies of plasma proteins have been carried out under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Columbia University. The preparations examined were kindly supplied by the Department of Physical Chemistry, Harvard Medical School.

with hydrogen sulfide, was at pH 3.5; it was concentrated to a small volume and cooled. Tyrosine crystallized as a mixture of the *l* and *dl* isomers (8). This was converted into the copper salts by treatment with basic copper carbonate in a volume calculated to be just sufficient to dissolve all

TABLE I

Composition of Bovine and Human Serum Albumin

Amino acid	Bovine	Human	No of residues per mole, mol. wt. = 70,000	
			Bovine	Human
	per cent	*per cent*		
Glutamic......	16.95	17.03	81	81
Aspartic......	10.25	9.77	54	52
Tyrosine......	5.53	4.73	21	18
Glycine......	1.96	1.60	18	15
Lysine.........	12.42		60	

TABLE II

*Analysis of Crystalline Bovine Serum Albumin**

Experiment No.	Amount of protein hydrolyzed (B)	Amino acid added			N^{15} excess in compound at successive stages of recrystallization (C)	Amino acid in protein $\left(\frac{C_0}{C} - 1\right) \times A\frac{100}{B}$
		Compound	Amount of *l*-amino acid (A)	N^{15} excess (C_0)		
	gm.		*mg.*	*atom per cent*	*atom per cent*	*per cent*
1	4.925	Glutamic acid	39.9	18.69	0.840, 0.857, 0.866	16.92
4	9.10	" "	94.7	18.69	1.091, 1.071, 1.079	16.97
1	4.925	Aspartic "	36.75	11.82	0.790, 0.800	10.35
4	9.10	" "	90.60	11.82	1.057, 1.051, 1.056	10.16
9	7.12	Tyrosine	63.37	6.85	0.954, 0.949, 0.945	5.53
1	4.925	Glycine	307.1	3.13	2.38, 2.43, 2.37	1.95
4	9.10	"	300.7	3.01	1.92, 1.87, 1.87	1.96
9	7.12	Lysine	49.89	9.09	0.483, 0.488, 0.485	12.42

* Preparations "17" (used in Experiment 1) and "C. B. 25" (used in Experiments 4 and 9), from the Department of Physical Chemistry, Harvard Medical School (*cf.* (2)).

of the copper *dl*-tyrosine at 0° (6). The *l* salt which crystallized was freed from copper and the resulting *l*-tyrosine purified by recrystallization until the specific rotation of each sample conformed with the data of Stein, Moore, and Bergmann (8). The tyrosine values for bovine and human serum albumins, 5.53 and 4.73 per cent respectively, found by the isotope

dilution method are in very good agreement with those of Brand *et al.* (2), 5.49 and 4.66 per cent respectively, determined by their photometric method (9).

Glutamic Acid—The barium salts of glutamic and aspartic acids were precipitated from the tyrosine filtrate. The glutamic acid, isolated as the hydrochloride from the dicarboxylic acid fraction, was recrystallized from 20 per cent hydrochloric acid under conditions such that at least twice the weight of *dl*-glutamic acid hydrochloride added to the protein hydrolysate

TABLE III

*Analysis of Crystalline Human Serum Albumin**

Experiment No.	Amount of protein hydrolyzed (B)	Amino acid added: Compound	Amino acid added: Amount of *l*-amino acid (A)	Amino acid added: N^{15} excess (C_0)	N^{15} excess in compound isolated at successive stages of recrystallization (C)	Amino acid in protein $\left(\frac{C_0}{C} - 1\right) \times A\frac{100}{B}$
	gm.		*mg.*	*atom per cent*	*atom per cent*	*per cent*
2	10.49	Glutamic acid	90.16	18.69	0.891, 0.884, 0.891	17.21
3	7.80	" "	89.37	18.69	1.183, 1.185, 1.187, 1.195	16.90
5	7.98	" "	86.80	18.69	1.115, 1.132, 1.136, 1 133, 1.139	16.90
7	5.86	" "	73.72	18.69	1.268, 1.281, 1.294	17.11
2	10.49	Aspartic "	84.20	11.82	0.873, 0.873, 0.876	10.05
3	7.80	" "	91.05	11.82	1.274, 1.275, 1.267, 1.261	9.70
5	7.98	" "	74 60	11.82	1.047, 1.064, 1.055, 1.053	9.57
5	7.98	Tyrosine	77.65	6.85	1.160, 1.158	4.78
7	5.86	"	379.8	1.025†	0 596, 0.595, 0.590, 0.600	4.68
5	7.98	Glycine	266.3	3.03†	2.06, 2.05, 2.05, 2.05	1.60
7	5.86	"	352.7	2.54†	1.99, 2.06, 2.05, 2.06	1.50

* Preparation "Com. 1" from the Department of Physical Chemistry, Harvard Medical School (*cf.* (2)).

† In these experiments glycine containing 33.8 atom per cent N^{15} excess and tyrosine containing 6.85 atom per cent N^{15} excess were diluted with known amounts of non-isotopic glycine and *l*-tyrosine.

would have remained in solution (5). The specific rotation, based on free glutamic acid, was $[\alpha]_D^{25} = +31.0° \pm 0.2°$ (2 to 3 per cent in 2.5 N HCl) for all samples.

The necessity of removing cystine prior to the precipitation of the barium dicarboxylates was demonstrated by the following observation. In the purification of glutamic acid hydrochloride from a hydrolysate of human serum albumin which had not been treated with cuprous oxide, the isotope content at successive stages of recrystallization was constant (0.736,

0.719, and 0.735 atom per cent N^{15} excess) and the nitrogen content was 7.7 per cent, from which it might have been concluded that the substance was pure *l*-glutamic acid hydrochloride. However, the specific rotation, even after five recrystallizations, was $-4.3°$ instead of $+31°$. The contaminant proved to be cystine dihydrochloride, the identity of which was established by the isolation of crystalline cystine by precipitation at pH 4 to 5. Its presence is explained by the high cystine content of serum albumin (2). It was found that the solubility of cystine dihydrochloride in 20 per cent hydrochloric acid at 0° is considerably lower than that of glutamic acid hydrochloride in the same solvent.[1]

Aspartic Acid—The filtrate from the glutamic acid hydrochloride was taken to dryness *in vacuo* and from the residue aspartic acid was isolated as the copper salt. *l*-Aspartic acid was regenerated from the recrystallized copper aspartate and repeatedly recrystallized. The specific rotation, based on free aspartic acid, was $[\alpha]_D^{25} = +25.3° \pm 0.2°$ (1.5 to 3 per cent in 2.5 N HCl) for all samples.

Glycine—From the filtrate of the barium salts of the dicarboxylates, freed from barium and alcohol and concentrated *in vacuo*, glycine was isolated as the trioxalatochromiate (10) and converted into the *p*-toluenesulfonyl derivative, m.p. 147–148°. The values for glycine are not as precise as those for the other amino acids, for the ratio of the isotope concentration of the added glycine to the isotope concentration of the isolated glycine is low (1). As the proteins contain but little glycine, it was necessary to add comparatively large amounts of glycine in order to isolate enough of the pure amino acid for the N^{15} determinations at successive stages of recrystallization.

Lysine—Lysine was isolated as the ε-monobenzoyl derivative according to the method of Kurtz (11) from a hydrolysate of bovine serum albumin from which cystine, tyrosine, and the leucine-phenylalanine fraction had been removed. The purity of the ε-benzoyl-*l*-lysine was established by nitrogen analysis and by constancy of isotope concentration and specific rotation on successive recrystallization. N = 11.1 per cent (calculated 11.2 per cent); $[\alpha]_D^{25} = +18.8° \pm 0.2°$ (2.3 per cent in N HCl).

The specific rotation in N HCl of the isolated ε-benzoyl-*l*-lysine was constant during five recrystallizations and the same as that of a sample prepared from pure *l*-lysine. The value observed for the reference sample in 50 per cent acetic acid was $+11.4°$, in contrast to $+27.2°$ reported by Goldschmidt and Kinsky (12). The reason for this discrepancy is not clear.

[1] Solubility determinations were carried out on cystine dihydrochloride and glutamic acid hydrochloride in 6.05 N hydrochloric acid at 0°; the solubilities were 0.51 and 0.90 gm. per 100 gm. of solution of cystine dihydrochloride and glutamic acid hydrochloride respectively. The solubility of glutamic acid hydrochloride is the same as that found by Graff, Rittenberg, and Foster (5).

Non-Conversion of Serine to Glycine—The possibility that glycine might be formed, by a decomposition of serine in acid solution analogous to that known to occur in an alkaline medium (13), was investigated by the isotope dilution method. To 990 mg. of serine, 500 mg. of isotopic glycine containing 1.095 atom per cent N^{15} excess were added and the mixture was refluxed for 17 hours with 100 cc. of 20 per cent hydrochloric acid. From this mixture glycine was precipitated with trioxalatochromiate and purified as the *p*-toluenesulfonyl derivative. The isotope value of the isolated glycine was found to be 1.091 atom per cent N^{15} excess. Since no significant dilution of the added glycine had occurred, it was concluded that none of the glycine in the protein hydrolysate was derived from serine.

SUMMARY

The isotope dilution method was employed to determine the amounts of glutamic acid, aspartic acid, tyrosine, and glycine yielded by crystalline bovine and human serum albumins, and of lysine yielded by bovine serum albumin.

It was found that cystine dihydrochloride is much less soluble in 20 per cent hydrochloric acid than glutamic acid hydrochloride, and in proteins containing appreciable amounts of cystine glutamic acid hydrochloride is very likely to be contaminated unless the cystine is first removed.

Conversion of serine to glycine does not occur under the conditions for acid hydrolysis of proteins.

BIBLIOGRAPHY

1. Rittenberg, D., and Foster, G. L., *J. Biol. Chem.*, **133**, 737 (1940).
2. Brand, E., Kassell, B., and Saidel, L. J., *J. Clin. Invest.*, **23**, 437 (1944).
3. Erlenmeyer, E., Jr., *Ber. chem. Ges.*, **35**, 3769 (1902).
4. Schoenheimer, R., and Ratner, S., *J. Biol. Chem.*, **127**, 301 (1939). Weissman, N., and Schoenheimer, R., *J. Biol. Chem.*, **140**, 779 (1941).
5. Graff, S., Rittenberg, D., and Foster, G. L., *J. Biol. Chem.*, **133**, 745 (1940).
6. Foster, G. L., *J. Biol. Chem.*, **159**, 431 (1945).
7. Bailey, K., Chibnall, A. C., Rees, M. W., and Williams, E. F., *Biochem. J.*, **37**, 360 (1943).
8. Stein, W. H., Moore, S., and Bergmann, M., *J. Am. Chem. Soc.*, **64**, 724 (1942).
9. Kassell, B., and Brand, E., *J. Biol. Chem.*, **125**, 115 (1938).
10. Bergmann, M., and Fox, S. W., *J. Biol. Chem.*, **109**, 317 (1935).
11. Kurtz, A. C., *J. Biol. Chem.*, **140**, 705 (1941).
12. Goldschmidt, S., and Kinsky, A., *Z. physiol. Chem.*, **183**, 244 (1929).
13. Daft, F. S., and Coghill, R. D., *J. Biol. Chem.*, **90**, 341 (1931).

THE EFFECT OF REPRODUCTION AND ESTROGEN ADMINISTRATION ON THE PARTITION OF CALCIUM, PHOSPHORUS, AND NITROGEN IN PIGEON PLASMA

By MARGARET R. McDONALD and OSCAR RIDDLE

(From the Department of Genetics, Carnegie Institution of Washington, Cold Spring Harbor, New York)

(Received for publication, April 4, 1945)

In oviparous vertebrates each period of egg production is associated with large increases in plasma calcium and phosphorus. Similar and even greater increases in these plasma components result from the administration of gonadotropin or estrogens, but not of androgens. The literature on this subject has been recently reviewed by Campbell and Turner (1), Riddle (2), and Gardner and Pfeiffer (3). Small increases in the plasma protein of fowl during egg production have also been reported (4–6).

A large part of the augmented phosphorus in the breeding females of birds, reptiles, and fishes (7–9), and in hens injected with gonadotropin (10), is due to enormous increases in the concentration of a phosphoprotein, serum vitellin, which is almost non-existent in the males and non-breeding females of these species. The effect of administered estrogens on the amount of serum vitellin has apparently not been studied (see "*Addendum*"). The remainder of the augmented phosphorus is due to increased inorganic and lipid phosphorus (7–10); it has been shown that both of these components can be raised by the injection of gonadotropin (10, 11) or estrogen (12–15).

The increment in plasma calcium due to egg laying (4, 16–18) or estrogen treatment (19) occurs in the non-ultrafiltrable fraction. Various suggestions have been made regarding the nature of the compound or compounds responsible for the binding of the extra calcium, but the available facts do not provide a definite solution of this problem. The several suggestions have been summarized and extended by Greenberg *et al.* (6). Their data for the laying hen indicate that part of the increment in non-diffusible calcium can be accounted for by the formation of a colloidal form of calcium phosphate and that the calcium-combining capacity of serum vitellin is sufficiently high to account for the remainder. Riddle and McDonald (19) found that one-third of the non-ultrafiltrable calcium in the plasma of estrogen-injected pigeons could be colloidal calcium phosphate.

The investigation reported here is primarily a study of the partition of those plasma components which might be capable of binding the large estrogen-induced increases in calcium. Changes in the various calcium, phosphorus, and nitrogen components of plasma have been studied (*a*)

during the normal reproductive cycle of female pigeons and (*b*) under the influence of injected estrogens in mature and immature, normal, fasted, parathyroidectomized, and hypophysectomized pigeons of both sexes. The correlations between the various constituents, in plasmas obtained under these extremely diversified conditions, should indicate which components are responsible for binding the calcium.

Materials and Procedures

Most of the pigeons utilized in this study were white Carneaux, purchased from the Palmetto Pigeon Plant, Sumter, South Carolina. Some birds of various races from our own colony were also used; the ratio of colony birds to white Carneaux was almost constant in the various comparative tests. The birds were ordinarily given a mixed grain diet consisting of cracked yellow corn, peas, kafir and wheat, and a commercial grit containing oyster shell, calcite, sand, salt, charcoal, and Venetian red. Those studied during the reproductive cycle were restricted to grains (corn and kafir) of low calcium content during the 5 days previous to sampling. This procedure was preferred to a final complete fast of 24 hours, since, unless special precautions are taken, some such complete fasts can be of 36 instead of the desired 24 hours. The birds were studied at all seasons of the year, and heat was provided in glass-sided houses during the winter.

The various phases of the reproductive cycle in pigeons were determined as follows. Accurate records of the time of egg laying were kept. In pigeons the approximate time required to develop the two egg yolks, the hours at which each ovum leaves the ovary (ovulation), and the time required for the egg to traverse the oviduct (43 hours) are fairly well established. The time since the last previous ovulation was therefore readily calculated. The weight (and race) of the birds, the diameter of the largest ovum, and the weight of the oviduct provided data from which time till the next ovulation was calculated.

Parathyroidectomy was performed by the method of Smith (20) and hypophysectomy (anterior lobe only) by the method of Schooley (21). The care and treatment of the birds operated on have been previously described (22).

The authors are indebted to Guinevere C. Smith for the operations and also for the care of the treated birds. Estradiol benzoate and androstenedione were obtained from the Schering Corporation through the courtesy of Dr. Erwin Schwenk. Diethylstilbestrol was contributed by Sharp and Dohme, Inc., and dihydrotachysterol (A. T. 10) by the Winthrop Chemical Company, Inc. All of these compounds were injected intramuscularly in volumes varying from 0.1 to 0.3 ml.

Blood samples were taken 24 hours after the last injection. The birds

had to be sacrificed to obtain the volume of blood necessary for all of the desired analyses. They were bled first from the radial arteries of each wing and immediately thereafter from the left ventricle, heparin being used as an anticoagulant. The blood obtained (15 to 20 ml.) was centrifuged immediately and the plasma analyzed as follows: (*a*) 1 or 2 ml. of plasma were diluted with distilled water to 5 ml.; 5 ml. of 10 per cent trichloroacetic acid were then added, and the mixture left at room temperature for 10 minutes and centrifuged. Aliquots of the supernatant were analyzed for calcium, inorganic phosphorus, and acid-soluble phosphorus. The precipitate was analyzed for protein nitrogen, lipid phosphorus, and protein phosphorus. (*b*) 0.5 ml. of plasma was diluted 25 times with water, and aliquots measured for total phosphorus and nitrogen. (*c*) The remainder of the plasma was ultrafiltered and aliquots of the ultrafiltrate analyzed for calcium, inorganic phosphorus, and total phosphorus.

Analytical Methods

Ultrafiltration—Ultrafiltration was performed by the method of Greenberg and Gunther (23); viscose instead of collodion sacs were used. All ultrafiltrates were tested for protein and the few which gave positive results discarded. Further confirmation of the satisfactoriness of the ultrafiltrates was obtained by analyzing them for total phosphorus in addition to inorganic phosphorus. No ultrafiltrate was used in which the difference between these two analyses was significantly greater than the difference between the acid-soluble and inorganic phosphorus of the plasma.

All data for the ultrafiltrates were corrected for the volume of the plasma proteins with the value 0.75 for the specific volume of the plasma proteins (24). Non-ultrafiltrable data were obtained as differences between the total and ultrafiltrable values.

Calcium—Total and ultrafiltrable calcium was precipitated as calcium phosphate by the method of Roe and Kahn (25) from 3 ml. of a 5 per cent trichloroacetic acid solution (containing approximately 0.06 mg. of calcium), with 0.5 ml. of 25 per cent sodium hydroxide and 0.5 ml. of 1.2 per cent trisodium phosphate. The precipitate was washed by the same method, and the calcium phosphate dissolved in 1.2 ml. of 60 per cent perchloric acid and analyzed for phosphate according to King (26).

Phosphorus—Laskowski's procedure (8) for the separation of the various phosphorus components was used, and aliquots of each phosphorus fraction analyzed by the method of King (26). In all samples analyzed the sum of the acid-soluble, lipid, and protein phosphorus was found to equal the total phosphorus.

Nitrogen—All nitrogen determinations were made by the micro-Kjeldahl method. The protein nitrogen values given are the sums of the nitrogen

found[1] in the alcohol-ether extracts of the trichloroacetic acid precipitate and that of the residue after extraction. No difference was found between the values thus obtained and those determined directly on the trichloroacetic acid precipitate. Protein nitrogen values have been corrected for lipid nitrogen with the equation, corrected protein N = observed protein N minus 0.5 lipid P. Non-protein nitrogen was determined as the difference between total nitrogen and uncorrected protein nitrogen. Lipid nitrogen is not included in this fraction (27).

Results

The changes in the partition of the various calcium, phosphorus, and nitrogen components of pigeon plasma found in female pigeons during the reproductive cycle and after the injection of estrogens (in mature and immature, male and female, normal, fasted, parathyroidectomized and hypophysectomized pigeons) are given in Fig. 1 and Table I. When differences due to age and the operative state of the birds are taken into consideration (19), it is evident that estrogens, whether naturally produced (during reproduction) or experimentally administered, probably effect no significant change in either the ultrafiltrable calcium or ultrafiltrable inorganic phosphorus content of pigeon plasma. They do, however, bring about large increases in the non-ultrafiltrable (colloidal plus non-ultrafiltrable-non-colloidal) calcium and non-ultrafiltrable inorganic phosphorus.[2] The individual data for these two components in all of the birds studied are shown in Fig. 2. A direct relationship apparently exists between their increased values, whether the increase results naturally or experimentally. The calculated lines of regression for each of the various groups were found to be practically identical. Calculation of the correlation coefficient for the 102 birds which showed varying degrees of hypercalcemia (12 to 60 mg. of total calcium per 100 ml. of plasma) due to endogenous or injected estrogen gave the highly significant value, $r = 0.955 \pm 0.009$. The correlation for the 72 control birds with normal or low (due to operation) calcium was only $r = 0.566 \pm 0.087$. This may be due in part to inaccuracies resulting from the measurements when only small amounts of non-ultrafiltrable inorganic phosphorus are present. Greenberg *et al.* (6) noted, however, that, although there is a direct relationship between the amounts of serum calcium and inorganic phosphorus in fowl during the laying period, such a relationship is not apparent at other times.

[1] When the trichloroacetic precipitate of plasma was suspended in alcohol-ether to extract the phospholipids (7), it was found that approximately one-third of the protein nitrogen was also extracted. Unpublished experiments of the authors show this alcohol-ether-soluble fraction to be albumin.

[2] Low non-ultrafiltrable inorganic phosphorus values were found in the few birds dying of tetany when sampled. These analyses have been omitted in Fig. 2.

Various workers have shown that the non-ultrafiltrable inorganic phosphorus in plasma of laying hens and in mammals with experimental hypercalcemia exists in the form of colloidal calcium phosphate. The high correlation found above suggests that the non-ultrafiltrable inorganic phosphorus

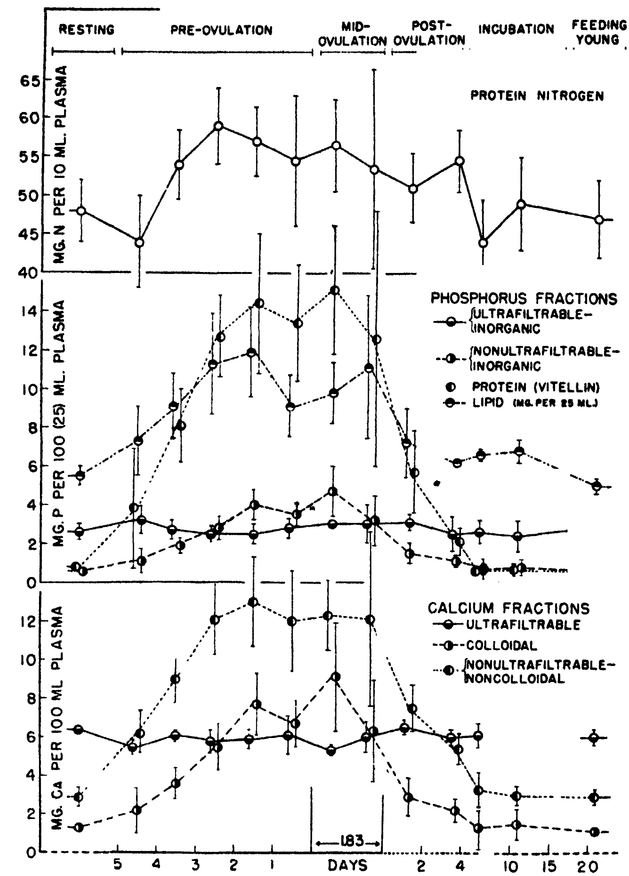


FIG. 1. Changes in the various calcium, phosphorus, and nitrogen components of plasma of female pigeons during the reproductive cycle. The circles represent the mean values obtained from four to eight (twenty-two for resting stage) birds; the vertical lines indicate plus or minus two standard errors of the mean; when no vertical line is shown, this line falls within the area of the circle.

of pigeon plasma is likewise present as colloidal calcium phosphate. The literature on the nature and formation of this colloid has been reviewed by Schmidt and Greenberg (28) and Greenberg (29). There is no direct method at present for determining the composition of this colloid. Green-

TABLE I

Partition of Calcium, Phosphorus, and Nitrogen in Plasma of Pigeons Given Various Treatments

The results are given in mg. per 100 ml. of plasma.

Daily treatment			Time from operation to killing	Age at killing	No. of birds	Sex	Calcium			Phosphorus				Protein N
								Non-ultrafiltrable		Inorganic				
Substance administered	Dosage	Duration					Ultrafiltrable	Colloidal $Ca_3(PO_4)_2$	Non-colloidal	Ultrafiltrable	Non-ultrafiltrable	Lipid	Protein	
Normal controls														
	mg.	*days*	*days*	*mos.*										
None..........				1.7	7	♀, ♂	5.6	2.5	2.7	5.1	1.3	23.3	0.8	365
"				2.5	10	♀, ♂	5.9	1.9	2.5	4.2	1.0	19.1	0.7	405
"				3.1	4	♂	6.3	1.7	2.5	4.0	0.9	18.7	0.7	439
"				Ad.	22	♀, ♂	6.4	1.2	3.0	2.6	0.6	21.8	0.8	481
Ca (+ 0.125 mg. A. T. 10)...........	200	15		2.8	8	♀, ♂	6.6	1.3	2.5	3.8	0.7	19.3	0.7	377
Alumina gel...	1000	19		2.9	3	♀, ♂	6.4	1.0	2.9	4.1	0.5	20.4	0.5	376
Parathyroidectomized controls														
Ca (+ 0.125 mg. A. T. 10).	200	9	9	2.3	4	♀, ♂	4.5	0.4	3.1	9.8	0.2	26.0	1.1	437
Ca (+0.125 mg. A. T. 10)....	200	14	14	Ad.	5	♀	5.0	1.9	2.8	6.6	1.0	23.7	1.0	503
Ca (+0.125 mg. A. T. 10)....	200	5	5	"	1*	♂	0.5*	0.4*	4.8	10.5*	0.2*	27.3	1.6	451
Alumina gel...	1000	20	20	3.2	3	♀, ♂	6.4	1.2	1.8	6.2	0.6	20.6	0.5	375
Hypophysectomized controls														
None..........			14	2.7	6	♀, ♂	5.7	1.6	1.8	4.2	0.8	10.5	0.5	312
Normal, from 118 hrs. before to 40 hrs. after ovulation														
None..........				Ad.	53	♀	5.9	5.8	10.6	2.8	3.0	38.7	10.9	543
Normal, injected with estrogen														
Estradiol benzoate†...	0.25	8		2.5	3	♀, ♂	7.4	2.9	4.8	3.9	1.5	24.1	2.9	397
Estradiol benzoate†...	0.5	5		2.3	7	♀, ♂	6.1	10.1	15.7	4.5	5.2	47.1	17.6	544
Estradiol benzoate†...	0.5	14		1.8	4	♀, ♂	5.1	6.8	12.3	4.7	3.5	39.7	14.0	523
Estradiol benzoate‡...	0.5	14		2.7	6	♀	6.4	12.4	18.6	2.8	6.4	45.3	21.1	599
Diethylstilbestrol†.....	0.2	15		Ad.	3	♂	6.5	4.1	10.9	3.9	2.1	42.5	9.8	558

TABLE I—*Concluded*

Daily treatment			Time from operation to killing	Age at killing	No. of birds	Sex	Calcium			Phosphorus				Protein N
Substance administered	Dosage	Duration					Ultrafiltrable	Non-ultrafiltrable		Inorganic		Lipid	Protein	
								Colloidal $Ca_3(PO_4)_2$	Non-colloidal	Ultrafiltrable	Non-ultrafiltrable			
Normal, injected with estrogen—*Continued*														
	mg.	*days*	*days*	*mos.*										
Estradiol benzoate†...	0.25	25		Ad.	2	♂	6.5	6.8	14.6	4.0	3.5	29.3	12.4	458
Estradiol benzoate‡...	0.5	6		"	6	♂	5.5	12.4	20.0	2.3	6.4	60.6	24.4	655
Fasted normal, injected with estrogen														
Estradiol benzoate†...	0.5	14		2.8	4	♂	5.7	14.7	21.3	2.9	7.6	39.3	26.9	619
Parathyroidectomized, injected with estrogen														
Estradiol benzoate†§..	0.25	15	17	2.5	2	♂	4.1	7.6	10.3	4.1	3.9	29.5	11.0	467
Estradiol benzoate†§..	0.25	15	17	2.5	2*	♀	1.4*	1.7*	9.8	11.2*	0.9*	40.1	10.3	497
Estradiol benzoate‡‖..	0.5	15	20	3.3	3	♀, ♂	6.6	17.0	28.7	4.0	8.8	55.5	34.4	703
Estradiol benzoate†§..	0.25	15	17	Ad.	3	♀	5.0	6.6	11.1	5.6	3.4	41.8	12.5	542
Estradiol benzoate†§..	0.25	15	17	"	1*	♀	0.2*	0.0*	19.7	12.1*	0.0*	66.7	20.5	636
Estradiol benzoate†§..	0.25	25	27	"	2	♂	4.3	3.7	6.4	6.1	1.9	23.8	6.4	404
Hypophysectomized, injected with estrogen														
Estradiol benzoate‡...	0.5	3	16	2.8	2	♀, ♂	6.1	7.4	9.7	3.6	3.8	30.2	12.1	401
Estradiol benzoate†...	0.5	14	15	2.7	2	♀, ♂	6.9	6.8	10.9	4.2	3.5	19.2	12.1	358
Normal, injected with androgen														
Androstenedione‡.......	3.0	15		2.7	3	♀	6.7	0.8	2.7	4.1	0.4	20.9	0.6	378

Ad. = adult.

* Birds dying in tetany when sampled.

† Dissolved in propylene glycol.

‡ Dissolved in sesame oil.

§ Plus 200 mg. of Ca + 0.125 mg. of A. T. 10 daily from time of operation.

‖ Plus 1 gm. of alumina gel daily from time of operation.

berg and Larson (30), using indirect methods, have assigned to it the composition $Ca_3(PO_4)_2$. With this formula the amounts of calcium bound as colloidal calcium phosphate have been calculated and the values given as colloidal calcium in Fig. 1 and Table I. *The term colloidal calcium is hereafter used to designate that part of the calcium which is bound as colloidal*

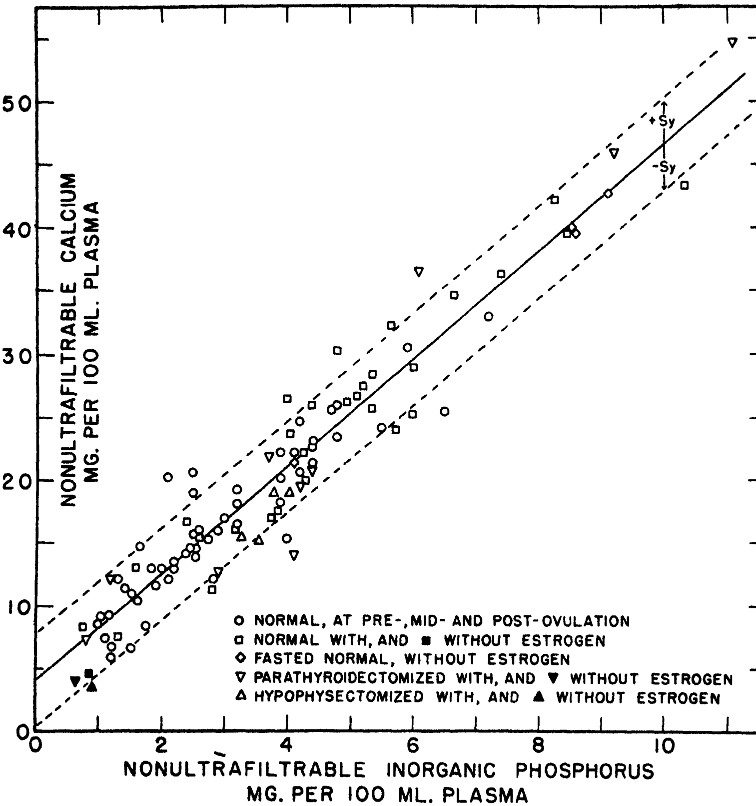


FIG. 2. Correlation between non-ultrafiltrable calcium (y) and non-ultrafiltrable inorganic phosphorus (x). The solid points are the means for each control group. The solid line represents the regression equation, $y = 4.22x + 4.17$. The dotted lines indicate one standard error of estimate ($S_y = 3.8$).

$Ca_3(PO_4)_2$; subtraction of colloidal calcium from total non-ultrafiltrable calcium yields the fraction non-ultrafiltrable-non-colloidal calcium. It can readily be seen that in all the birds studied only about one-third of the non-ultrafiltrable calcium can be accounted for as colloidal calcium phosphate.

Examination of the data in Fig. 1 and Table I shows that as the non-ultrafiltrable-non-colloidal calcium increases the lipid phosphorus also

increases. Drinker and Zinsser (31) noted that cephalin can combine with calcium, and estimated that 30 to 40 per cent of the bound calcium in normal mammalian sera could be united to this phospholipid. The limited amount of blood obtainable from a pigeon made direct determinations of cephalin, in addition to the other desired analyses, impracticable. Flock and Bollman (32), however, found that the administration of diethylstilbestrol

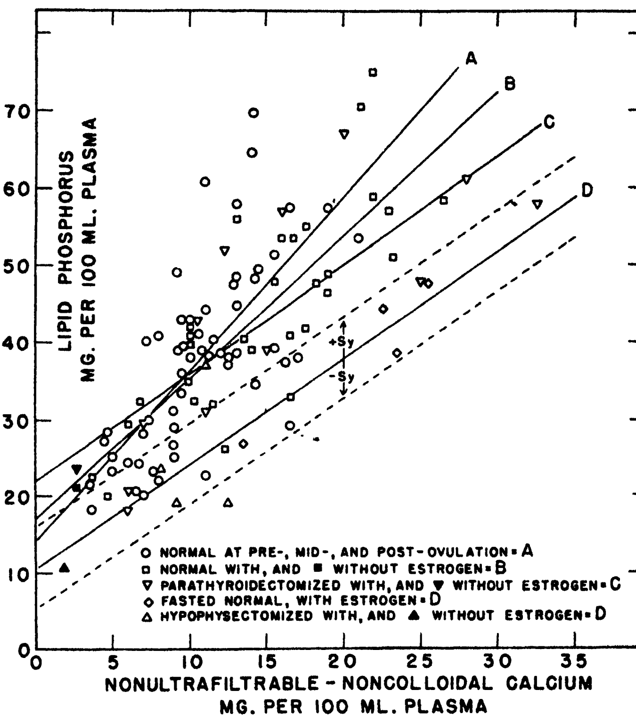


FIG. 3. Correlation between lipid phosphorus and non-ultrafiltrable-non-colloidal calcium. The solid lines represent the regression equations for each type of bird studied (Table II) and the solid points the means for each control group. The dotted lines indicate one standard error of the estimate ($S_y = 5.7$) for the hypophysectomized fasted pigeons.

increased both the cephalin and the choline-phospholipid content of cock plasma without materially altering their ratio. Fig. 3 and Table II, in which the individual data for lipid phosphorus and non-ultrafiltrable-non-colloidal calcium are given, show that, while there is some correlation between these components, the calculated lines of regression for the various types of pigeons studied do not coincide. This is particularly true for the hypophysectomized and fasted pigeons, and indicates that the non-ultra-

filtrable-non-colloidal calcium of pigeon plasma is probably not bound by phospholipid. The results of Greenberg *et al.* (6), although not conclusive, also provided evidence for the absence of a lipid-calcium compound in the serum of laying hens. The work of Taurog, Entenman, and Chaikoff (33), published since the completion of this study, casts doubt on the presence of cephalin in plasma. The degree of correlation ($r = 0.704 \pm 0.049$) found between the lipid phosphorus and non-ultrafiltrable-non-colloidal calcium can probably be regarded as a consequence of the fact that both are increased by the same provocative agent, estrogen.

It can be seen from Fig. 1 and Table I that the enormous increases in non-ultrafiltrable-non-colloidal calcium due to endogenous (at egg production) and administered estrogens are accompanied by only slight increases in protein nitrogen. These increases, if the protein remained unchanged in kind, should be capable of binding only 1 to 2 mg. of calcium, thus leaving more than 80 per cent of the non-ultrafiltrable-non-colloidal calcium unaccounted for. Furthermore, calculations of the correlation coefficient between the total protein nitrogen and the non-ultrafiltrable-non-colloidal calcium give the rather low value, $r = 0.696 \pm 0.051$.

TABLE II

Relation between Lipid Phosphorus (y) and Non-Ultrafiltrable-Non-Colloidal Calcium (x) in Normal, Fasted, Hypophysectomized, and Parathyroidectomized Pigeons Showing Hypercalcemia Due to Endogenous or Administered Estrogens

Type of pigeon	No. of birds	Regression equation, $y = mx + c$		Standard error of estimate, S_y	Correlation coefficient, r
		(m)	(c)		
Normal, near ovulation	53	2.24	14.0	8.8	0.69 ± 0.07
" estrogen-injected	31	1.83	17.1	7.6	0.81 ± 0.06
Parathyroidectomized, estrogen-injected	13	1.40	21.8	10.8	0.78 ± 0.12
Fasted normal, and hypophysectomized, estrogen-injected	8*	1.37	10.4	5.7	0.84 ± 0.10
All of above types	105				0.70 ± 0.05

* Four birds of each type.

Greenberg *et al.* (6) and Laskowski[3] have noted that the phosphoprotein, serum vitellin, probably has a much higher calcium-combining capacity than the usual serum proteins. This protein, as can be seen in Fig. 1 and Table I, is markedly increased by the administration of estrogens as well

[3] Laskowski, M., personal communication to the authors.

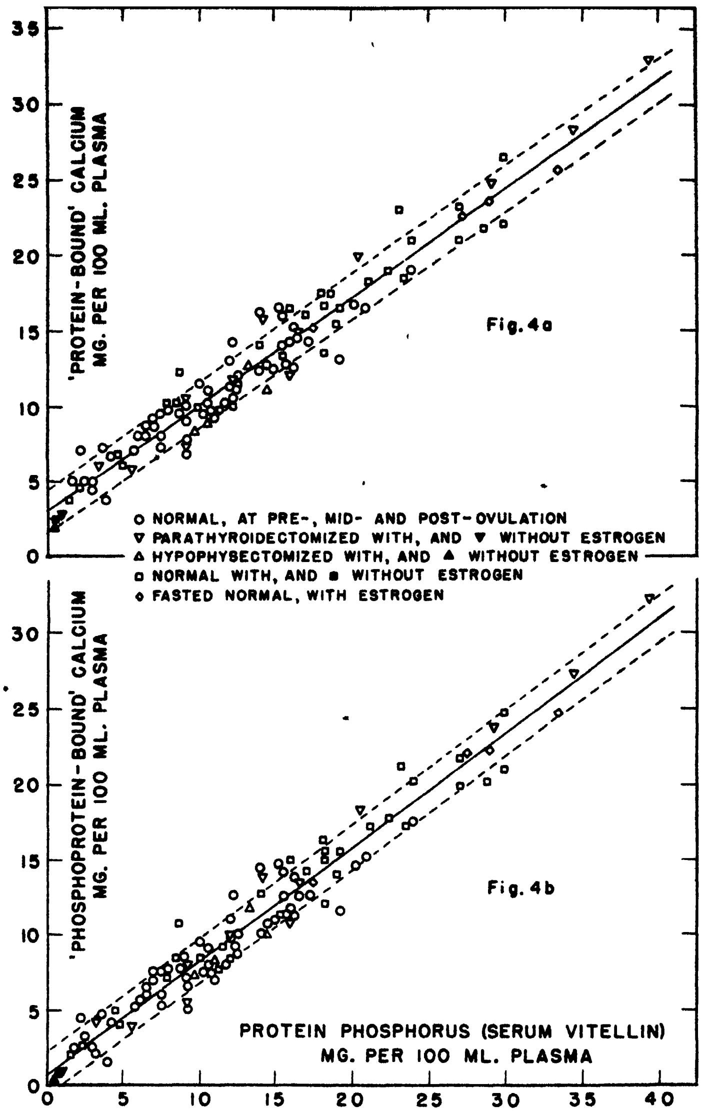


FIG. 4, *a*. Correlation between protein phosphorus (serum vitellin) and "protein-bound" (non-ultrafiltrable-non-colloidal) calcium. The solid line represents the regression equation, protein-bound Ca = 0.712 protein P + 2.99. The dotted lines indicate one standard error of estimate (S_y = 1.43).

FIG. 4, *b*. Correlation between protein phosphorus (serum vitellin) and "phosphoprotein-bound" (non-ultrafiltrable-non-colloidal calcium minus calcium bound to plasma proteins other than vitellin) calcium. The central line represents the regression equation, phosphoprotein-bound Ca = 0.751 protein P + 0.74. The dotted lines indicate one standard error of estimate (S_y = 1.47). In Figs. 4, *a* and 4, *b* the solid points are average values for each control group.

as during the egg production cycle, and the increase parallels the increase in non-ultrafiltrable-non-colloidal calcium. The individual data for these two components for all of the birds studied are given in Fig. 4, *a*, where, for convenience, the non-ultrafiltrable-non-colloidal calcium prematurely has been termed "protein-bound" calcium. It is evident that there is a marked relationship between the non-ultrafiltrable-non-colloidal calcium and the protein phosphorus content of pigeon plasma. Calculation of the correlation coefficient[4] between these components for the 53 reproducing pigeons (from 118 hours before to 40 hours after ovulation) and for the 52 estrogen-injected pigeons gives the extremely significant value, $r = 0.969 \pm 0.006$. The calculated lines of regression for the various types of pigeon were found to coincide, but they do not pass through the origin. These results suggest that part but not all of the non-ultrafiltrable-non-colloidal calcium is bound to the phosphoprotein, serum vitellin.

The data for normal pigeons (Table I) indicate that the magnitude of the calcium-combining capacity of the ordinary proteins of pigeon plasma is the same as that of mammalian plasma. Assuming this to be true, the amount of calcium bound by the plasma proteins other than vitellin can be calculated with the equation

$$Ca_{n\mathrm{VPr}} = 0.00527\ (\mathrm{TPr\ N} - 15.5\ \mathrm{Pr\ P})$$

in which $Ca_{n\mathrm{VPr}}$ = mg. calcium bound to protein other than vitellin
0.00527 = " " " by 1 mg. of protein nitrogen[5] (30)
TPr N = " total protein nitrogen
Pr P = " protein phosphorus
15.5 = factor to convert Pr P to Pr N[3]

Subtraction of $Ca_{n\mathrm{VPr}}$ from non-ultrafiltrable-non-colloidal calcium yields the fraction tentatively called "phosphoprotein-bound" calcium. When the data for this fraction are plotted against the data for protein phosphorus (serum vitellin), Fig. 4, *b*, the lines of regression for the various types of birds studied still coincide, but they now, within the limits of accuracy of the measurements, also pass through the origin. Statistical evaluation of the correlation between the "phosphoprotein-bound" calcium and the protein phosphorus for the 105 pigeons studied (with degrees of hypercalcemia varying from 12 to 60 mg. of total calcium per 100 ml. of plasma) yields the highly significant result, $r = 0.972 \pm 0.005$. It would thus seem that all of the non-ultrafiltrable-non-colloidal calcium in pigeon plasma which

[4] The correlation between these variables for the control pigeons has not been calculated, since the amounts of protein phosphorus in these birds are much too small to be measured with any degree of accuracy and such calculations would therefore be meaningless.

[5] Unpublished data of the authors show that the ratio of albumin to globulin in normal pigeon plasma is almost 1:1.

is not bound by the ordinary plasma proteins is bound by the phosphoprotein, serum vitellin. The calcium-binding capacity of the latter is very great. Calculations from the regression equation (Fig. 4, *b*) show that 1 gm. of serum vitellin (containing 0.95 to 0.99 per cent phosphorus[3]) can combine with more than 7 mg. of calcium, a value 8 to 9 times greater than that of the normal plasma proteins (30).

Computation of the data in Table I and Fig. 1 shows that, in both male and female pigeon plasma, the proteins other than vitellin decrease after estrogen treatment. This decrease, however, may be fallacious, since Laskowski's[3] values for the phosphorus (0.95 to 0.99 per cent) and nitrogen (15 per cent) content of vitellin, accepted for these calculations, may prove to be too low when more highly purified vitellin is prepared. If the decrease is real, it might suggest that serum vitellin is formed partly at the expense of the other plasma proteins. Laskowski (34) has presented indirect evidence indicating that the amount of globulin (other than vitellin) is smaller and the amount of albumin larger in the blood of laying hens. In the 73 control birds serum vitellin comprises only about 3 per cent of the total plasma proteins, while in the 53 birds in which ova were actively growing or being released it averaged 31 per cent; and in the 52 estrogen-injected birds it averaged 50 per cent.

The data on non-protein (total less protein) nitrogen and ester phosphorus (acid-soluble less inorganic) are very inaccurate, since they are obtained as small differences between two much larger values. No considerable change was found, however, in the concentration of these components in the plasma of pigeons at egg production or after the injection of estrogens. Mean values plus or minus the standard error (expressed as mg. per 100 ml. of plasma) for non-protein nitrogen and ester phosphorus of 26 $\pm$ 3.5 and 0.55 $\pm$ 0.039, respectively, were obtained for the control pigeons and of 24 $\pm$ 2.4 and 0.67 $\pm$ 0.038 for those subjected to estrogen (endogenous and administered) treatment. The slight decrease found for the non-protein nitrogen is probably not significant; the slight increase in ester phosphorus may or may not be significant.

The average values for the non-ultrafiltrable inorganic and protein phosphorus content of the plasma of the control pigeons were found to be 0.8 mg., and for lipid phosphorus 21 mg. per 100 ml. of plasma. Values as high as 11, 40, and 75 mg. per 100 ml. of plasma for the non-ultrafiltrable inorganic, protein, and lipid phosphorus, respectively, were obtained for the estrogen-treated pigeons. The changes in the distribution of these phosphorus fractions, as influenced by estrogens, are summarized in Table III. While lipid phosphorus accounts for 93 per cent of the non-ultrafiltrable phosphorus in the plasmas of the control pigeons, it accounts for only 56 per cent (average value for 97 pigeons) of the estrogen-induced increase in

non-ultrafiltrable phosphorus; protein phosphorus (serum vitellin) is responsible for 36 per cent, and colloidal (non-ultrafiltrable inorganic) phosphorus for the remaining 8 per cent.

The average value for the non-ultrafiltrable calcium content of the plasma of the control pigeons was found to be 4.2 mg. per 100 ml. of plasma. Increases of 2 to 29 mg. per 100 ml. of plasma were found during the reproductive cycle and of 3 to 50 mg. after the injection of estrogens. The augmented values are due to increments in colloidal calcium phosphate (1 to 19 mg. per 100 ml. of plasma) and phosphoprotein-bound calcium (1 to 31 mg. per 100 ml. of plasma). Fig. 5 analyzes the changes in the partition of the non-ultrafiltrable calcium in these estrogen-induced (endogenous and administered) hypercalcemias. As the non-ultrafiltrable calcium increases, the percentage of non-ultrafiltrable calcium bound as

TABLE III

Estrogen-Induced Changes in Partition of Phosphorus in Pigeon Plasma

Pigeons	No. of birds	Non-ultrafiltrable phosphorus*						
		Total	Colloidal†		Lipid		Protein	
		mg. per 100 ml. plasma	*mg. per 100 ml. plasma*	*per cent of total*	*mg. per 100 ml. plasma*	*per cent of total*	*mg. per 100 ml. plasma*	*per cent of total*
Control	67‡	23.0	0.8	3.5	21.4	93.0	0.8	3.5
Near ovulation	53	52.6	3.0	5.7	38.7	73.6	10.9	20.7
Estrogen-injected	44‡	64.8	4.4	6.8	43.8	67.6	16.6	25.6

* Mean values.
† Non-ultrafiltrable inorganic phosphorus.
‡ Hypophysectomized and fasted pigeons omitted.

colloidal calcium phosphate remains practically constant, but the percentage bound to the phosphoprotein (serum vitellin) increases rapidly, while the percentage bound to the plasma proteins other than vitellin decreases precipitously.

Fig. 1 shows that the various plasma calcium and phosphorus fractions which increase in pigeons during the reproductive cycle all follow the pattern previously observed by Riddle and associates for total plasma calcium (35, 36) and plasma fat (37). Increased values are already evident 5 days before ovulation of the first ovum. Maximal values are attained from 2.5 to 1.5 days before the ovulation of the first ovum; high values are more or less sustained until just prior to the ovulation of the second ovum, at which time there is a precipitous drop, and by 5 days after the second ovulation the values are again normal.

It is evident from Table I that neither fasting, parathyroidectomy, nor

hypophysectomy significantly modified the values for non-ultrafiltrable calcium (either colloidal or protein-bound), non-ultrafiltrable inorganic phosphorus, lipid phosphorus, or protein phosphorus resulting in pigeon plasma from the administration of estrogens. Similar results have been obtained by Riddle and associates for estrogen-increased total plasma calcium (22, 38) and fat (37) and for estrogen-induced endosteal bone formation (22) in pigeons. War time conditions have prevented the authors from seeing copies of publications of Benoit *et al.* in which they apparently have reported that parathyroidectomy prevents (39, 40), and hypophy-

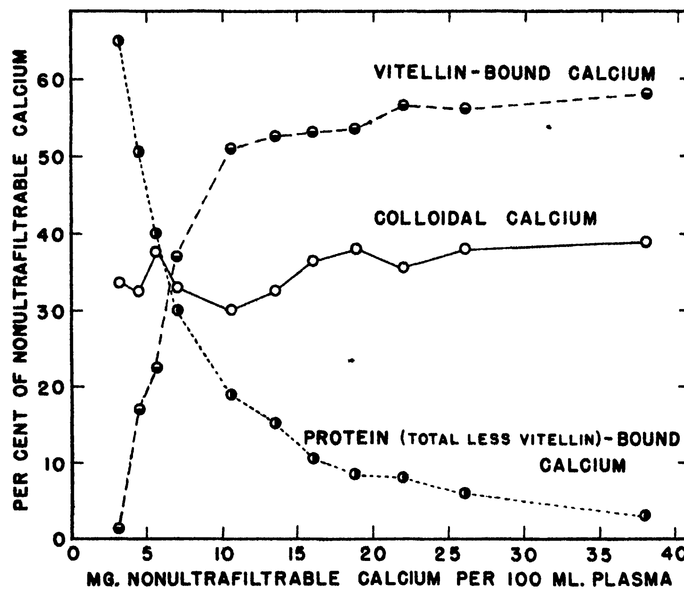


FIG. 5. Analysis of the partitions of non-ultrafiltrable calcium in pigeon plasma on a percentage basis. Each point represents the average of ten to twenty-nine determinations.

sectomy reduces by one-half (41), the increase in blood calcium in drakes caused by the injection of estradiol propionate.

It can also be seen from Table I that in normal pigeons neither the androgen, androstenedione, nor the therapeutic agents (A. T. 10 + calcium gluconate, or alumina gel) used to maintain life in parathyroidectomized pigeons significantly altered any of the components studied. Supplementary data confirming this fact are given in Table IV. The decrease in calcium and the increase in inorganic phosphorus found in parathyroidectomized pigeons are, however, partially reversed by the administration of alumina gel or of dihydrotachysterol (A. T. 10) and calcium gluconate.

TABLE IV

Effect of Daily Administration of Androstenedione, Alumina Gel, and A. T. 10 Plus Calcium Gluconate on Calcium, Phosphorus, and Nitrogen Components of Pigeon Plasma

The results are given in mg. per 100 ml. of plasma.

Pigeons	Daily treatment	Days* treated	No. of birds	Total calcium	Phosphorus			Protein nitrogen
					Inorganic	Lipid	Protein	
Normal	None		21	10.5	5.5	20.4	0.7	398
	3 mg. androstenedione	2	3	10.2	5.1	22.6	0.5	379
		6	3	10.1	4.8	19.3	0.4	360
		15	3	10.2	4.5	20.9	0.6	378
	1 gm. alumina gel	12	3	10.4	5.2	22.4	0.7	388
		19	3	10.3	4.6	20.4	0.5	376
	0.125 mg. A. T. 10 + 0.2 gm. Ca (as gluconate)	2	3	11.1	5.3	21.6	0.7	414
		12	5	10.8	3.9			
		15	3	10.7	4.6	21.3	0.7	414
		19	5	10.2	4.3	18.1	0.7	354
Parathyroidectomized	1 gm. alumina gel	1†	8	4.7	8.5	19.5	0.6	465
		4	4	7.5	6.4	23.0	0.7	400
		6	4	8.4	4.5	23.8	0.6	478
		13	8	9.7	5.3	21.5	0.6	380
		16–28	4	9.4	6.0	22.5	0.6	403

* Samples of 3 to 4 ml. of blood were taken from the wing vein of each bird two to four times during the course of the experiment.

† Day after operation.

DISCUSSION

Chargaff (42) has shown that the vitellin of egg yolk is a lipovitellin complex containing about 18 per cent phosphatides, the composition of which is essentially the same as that of the phosphatides occurring in egg yolk in the free state. Laskowski has been unable to prepare serum vitellin free from phospholipid.[3] Calculation of the correlation coefficient between lipid and protein phosphorus for the 105 birds in which these components were increased by endogenous or administered estrogens gives the value, $r = 0.630 \pm 0.060$. This correlation might indicate union between protein phosphorus and part of the phospholipid, but it can also be regarded as a consequence of the simultaneous increase of both lipid and protein phosphorus under the influence of the same provocative agent (estrogen).

Benjamin and Hess (43) developed an analytical procedure (combining ultrafiltration and adsorption with barium sulfate) to divide the plasma

calcium into at least four fractions: filtrable ionic calcium, a filtrable adsorbable calcium-phosphorus complex, an unknown non-filtrable adsorbable complex, and protein-bound (non-filtrable-non-adsorbable) calcium. Their procedure has been widely used by others, but the possibility that protein also is adsorbed has apparently been neglected. Data obtained on pigeon plasma with their procedure are given in Table V; adsorbed serum vitellin and phospholipid, in addition to adsorbed calcium and inorganic phosphorus, were measured. The results show that, as was found by Benjamin and Hess (43), part of both ultrafiltrable and non-ultrafiltrable calcium

TABLE V

Comparison of Total and Barium Sulfate-Adsorbed Calcium and Phosphorus Fractions in Plasma of Pigeons

Fraction	Control pigeons*		Pigeons with hypercalcemia†	
	mg. per 100 ml. plasma	*per cent adsorbed*	*mg. per 100 ml. plasma*	*per cent adsorbed*
Total ultrafiltrable inorganic phosphorus	3.3		3.2	
Adsorbed " " "	2.6	79	2.5	78
Total non-ultrafiltrable " "	0.7		5.3	
Adsorbed " " "	0.2	29	1.8	34
Total lipid phosphorus	17.9		42.3	
Adsorbed lipid phosphorus	0.3	2	2.0	5
Total protein phosphorus	0.5		18.9	
Adsorbed " "	0.2	40	3.5	19
Total ultrafiltrable calcium	6.1		6.1	
Adsorbed " "	3.4	56	3.2	52
Total non-ultrafiltrable calcium	3.9		27.5	
Adsorbed " "	1.9	49	6.7	24
Total non-ultrafiltrable-non-colloidal calcium	2.5		17.3	
Adsorbed " "	1.5	60	3.2	19

* Average for eleven individuals.
† Average for twenty individuals.

and inorganic phosphorus is adsorbed by barium sulfate; but part of the phosphoprotein also is adsorbed. It is therefore impossible to distinguish, by the procedure of Benjamin and Hess, protein-bound calcium from the non-filtrable adsorbable complex. The results obtained in Table V are in better agreement with the view that there is a partial adsorption of all of the non-ultrafiltrable calcium fractions rather than a complete adsorption of one or more specific fractions.

Riddle (44) in 1927 noted the probability that the ovarian hormone, which is responsible for the cyclic growth of the oviduct, is also responsible for the increased plasma calcium, phosphorus, and fat found during the

egg-production cycle of pigeons. Almost all of the data published since that time have pointed to estrogens as the provocative agent for increases in these constituents in the blood of oviparous vertebrates during the reproductive cycle. The data presented here show that all of the changes in the partition of the various calcium, phosphorus, and nitrogen fractions which occur in the plasma of female pigeons at or near egg production can be duplicated by the injection of estrogens; and estrogens are effective in mature and immature, normal, parathyroidectomized, and hypophysectomized pigeons of both sexes.

SUMMARY

Changes in the partition of the various calcium, phosphorus, and nitrogen components of plasma have been studied (*a*) in female pigeons during the reproductive cycle and (*b*) in male and female, immature and mature, normal, fasted, parathyroidectomized, and hypophysectomized pigeons injected with estrogens.

No significant differences occur in either ultrafiltrable calcium, ultrafiltrable inorganic phosphorus, or non-protein nitrogen during the reproductive cycle or after the injection of estrogens.

Non-ultrafiltrable calcium, non-ultrafiltrable inorganic phosphorus, lipid phosphorus, and protein phosphorus increase markedly from 4 days before the ovulation of the first egg until 2 days after the ovulation of the second (last) egg. Similar, and even greater, increases result from the administration of estrogens in fasted, parathyroidectomized, hypophysectomized, and normal pigeons. Small increases in the plasma protein nitrogen were noted under these conditions.

A direct relationship ($r = 0.955 \pm 0.009$) was found between non-ultrafiltrable calcium and non-ultrafiltrable inorganic phosphorus in plasmas of estrogen-treated (endogenous and administered) pigeons. The non-ultrafiltrable inorganic phosphorus is probably a colloidal form of calcium phosphate.

A direct relationship ($r = 0.969 \pm 0.006$) was also noted between non-ultrafiltrable-non-colloidal calcium and protein phosphorus in pigeons with hypercalcemia due to reproduction or to injected estrogen.

The calcium-combining capacity of the phosphoprotein, serum vitellin, is 8 to 9 times greater than that of other plasma proteins.

Increments in both colloidal calcium phosphate and vitellin-bound calcium account for the increased non-ultrafiltrable calcium found during reproduction and after estrogen injection. In 72 control pigeons 36 per cent of the non-ultrafiltrable calcium occurred as colloidal calcium phosphate, 12 per cent was bound to vitellin, and 52 per cent was bound to plasma proteins other than vitellin. In 102 estrogen-treated (endog-

enous and administered) pigeons, however, 37 per cent of non-ultrafiltrable calcium was in the form of colloidal calcium phosphate, only 8 per cent was bound to plasma proteins other than vitellin, and 55 per cent was bound to serum vitellin.

Barium sulfate partially adsorbs both colloidal calcium phosphate and phosphoprotein-bound calcium; it does not separate satisfactorily the various non-ultrafiltrable calcium fractions.

Androstenedione (an androgen) had no significant effect on any of the calcium, phosphorus, and nitrogen components studied.

Addendum—After this manuscript was completed the authors had opportunity to see a manuscript (soon to be published) by Dr. Walter Fleischmann and Dr. Ilse A. Fried. These workers found that the injection of estradiol dipropionate into normal immature chicks of both sexes results in large parallel increases in serum calcium, inorganic phosphorus, lipid phosphorus, and protein phosphorus (serum vitellin). Moreover, when they injected thyroxine and estradiol dipropionate simultaneously into normal chicks no increase in these blood constituents resulted, although the weight of the oviduct was still increased. They suggest that thyroxine prevents the estrogen-induced increase in serum calcium indirectly either by inhibiting the formation of, or causing the destruction of the organic phosphorus compounds necessary to bind the augmented calcium.

BIBLIOGRAPHY

1. Campbell, I. L., and Turner, C. W., *Univ. Missouri, Res. Bull.* *352*, 93 (1942).
2. Riddle, O., *Endocrinology*, **31**, 498 (1942).
3. Gardner, W. U., and Pfeiffer, C. A., *Physiol. Rev.*, **23**, 139 (1943).
4. Benjamin, H. R., and Hess, A. F., *J. Biol. Chem.*, **103**, 629 (1933).
5. Rokhlina, M. L., *J. Physiol.*, U. S. S. R., **16**, 937 (1933); *Problèmes biol. méd.*, Moscow, 450 (1935).
6. Greenberg, D. M., Larson, C. E., Pearson, P. B., and Burmester, B. R., *Poultry Sc.*, **15**, 483 (1936).
7. Roepke, R. R., and Hughes, J. S., *J. Biol. Chem.*, **108**, 79 (1935).
8. Laskowski, M., *Biochem. Z.*, **275**, 293 (1935).
9. Laskowski, M., *Biochem. Z.*, **284**, 318 (1936).
10. Laskowski, M., *Biochem. J.*, **32**, 1176 (1938).
11. Entenman, C., Lorenz, F. W., and Chaikoff, I. L., *J. Biol. Chem.*, **126**, 133 (1938).
12. Landauer, W., Pfeiffer, C. A., Gardner, W. U., and Shaw, J. C., *Endocrinology*, **28**, 458 (1941).
13. Lorenz, F. W., Chaikoff, I. L., and Entenman, C., *J. Biol. Chem.*, **126**, 763 (1938).
14. Landauer, W., Pfeiffer, C. A., Gardner, W. U., and Man, E. V., *Proc. Soc. Exp. Biol. and Med.*, **41**, 80 (1939).
15. Entenman, C., Lorenz, F. W., and Chaikoff, I. L., *J. Biol. Chem.*, **134**, 495 (1940).
16. Laskowski, M., *Biochem. Z.*, **260**, 230 (1933).
17. Correll, J. T., and Hughes, J. S., *J. Biol. Chem.*, **103**, 511 (1933).
18. Taylor, M. W., and Russell, W. C., *J. Agr. Res.*, **51**, 663 (1935).
19. Riddle, O., and McDonald, M. R., *Endocrinology*, **36**, 48 (1945).
20. Smith, G. C., *Anat. Rec.*, in press (1945).
21. Schooley, J. P., *Endocrinology*, **25**, 372 (1939).
22. Riddle, O., Rauch, V. M., and Smith, G. C., *Endocrinology*, **36**, 41 (1945).

23. Greenberg, D. M., and Gunther, L., *J. Biol. Chem.*, **85**, 491 (1929–30).
24. Svedberg, T., and Sjögren, B., *J. Am. Chem. Soc.*, **50**, 3321 (1928).
25. Roe, J. H., and Kahn, B. S., *J. Biol. Chem.*, **81**, 1 (1929).
26. King, E. J., *Biochem. J.*, **26**, 292 (1932).
27. Peters, J. P., and Van Slyke, D. D., Quantitative clinical chemistry, Methods, Baltimore, 536 (1932).
28. Schmidt, C. L. A., and Greenberg, D. M., *Physiol. Rev.*, **15**, 297 (1935).
29. Greenberg, D. M., Annual review of biochemistry, Stanford University, **8**, 269 (1939).
30. Greenberg, D. M., and Larson, C. E., *J. Phys. Chem.*, **43**, 1139 (1939).
31. Drinker, N., and Zinsser, H. H., *J. Biol. Chem.*, **148**, 187 (1943).
32. Flock, E. V., and Bollman, J. L., *J. Biol. Chem.*, **144**, 571 (1942).
33. Taurog, A., Entenman, C., and Chaikoff, I. L., *J. Biol. Chem.*, **156**, 385 (1944).
34. Laskowski, M., *Arch. biochem.*, **4**, 41 (1944).
35. Riddle, O., and Reinhart, W. H., *Am. J. Physiol.*, **76**, 660 (1926).
36. Riddle, O., Rauch, V. M., and Smith, G. C., *Anat. Rec.*, **90**, 295 (1944).
37. Riddle, O., Senum, T., and Rauch, V. M., *Carnegie Inst. Washington, Pub. No. 569*, in press.
38. Riddle, O., and Dotti, L. B., *Proc. Am. Phil. Soc.*, in press (1945).
39. Benoit, J., Grangaud, R., and Messerschmidt, J., *Compt. rend. Soc. biol.*, **135**, 1396 (1941).
40. Benoit, J., Fabiani, G., Grangaud, R., and Clavert, J., *Compt. rend. Soc. biol.*, **135**, 1606 (1941).
41. Benoit, J., Clavert, J., and Fabiani, G., *Compt. rend. Soc. biol.*, **136**, 571 (1942).
42. Chargaff, E., *J. Biol. Chem.*, **142**, 491 (1942).
43. Benjamin, H. R., and Hess, A. F., *J. Biol. Chem.*, **100**, 27 (1933).
44. Riddle, O., *Proc. Am. Phil. Soc.*, **66**, 497 (1927).

THE DETERMINATION OF SERUM PROTEIN CONCENTRATION WITH A GRADIENT TUBE

By OLIVER H. LOWRY AND THOMAS H. HUNTER

(*From the Division of Nutrition and Physiology, The Public Health Research Institute of the City of New York, Inc., and the Department of Medicine, College of Physicians and Surgeons, Columbia University, Presbyterian Hospital, New York*)

(Received for publication, April 13, 1945)

The specific gravity of serum has been shown to be a reliable index of serum protein concentration (1, 2). The methods which appear to be best suited for the rapid determination of serum specific gravity are the falling drop method of Barbour and Hamilton (3), the copper sulfate procedure of Phillips *et al.* (4), and the gradient tube method of Linderstrøm-Lang. Although the use of the gradient tube for very precise specific gravity measurements has been carefully described (5), there have been but brief references to the adaptation of this method to the rapid measurement of serum or plasma specific gravity (6–8).

The simplicity, convenience, speed, and accuracy of the gradient tube method recommend it not only for routine hospital use and experimental purposes, but the method has been found to be almost ideally suited for large scale population studies of protein nutrition. For such survey work the fact that only 2 or 3 c.mm. of serum are required is particularly advantageous. It appears, therefore, desirable to describe the method more fully, and to give the results of 240 comparisons of serum protein values determined by the gradient tube with the protein concentrations calculated from the total nitrogen (Kjeldahl).

Details of collecting serum from the finger and comparisons between serum protein values obtained from finger, ear, and venous blood will be given, and the effects of storage, etc., will be discussed.

Method

Principle—By the use of mixtures of different proportions of a heavy and a light organic liquid, a linear density gradient is established in a graduated cylinder. Droplets of serum, 2 to 4 c.mm. in size, are allowed to fall into this gradient, and they come to rest upon reaching a point having a density exactly equal to their own. The density, and hence the protein concentration, are calculated graphically by interpolation from the position of droplets of salt solutions of known density. To prevent disturbances of the gradient tube by convection currents, the cylinder is installed in a jar of water.

Materials—

1. *Gradient tube and water jacket* (Fig. 1), a special 500 ml. graduated cylinder (*A*) fitted inside a larger cylinder (*B*). (This may be obtained from Eimer and Amend, New York, or may be easily constructed in the laboratory from readily available material; see Lowry and Hastings (7).)

2. *Gradient Solution A*, 100 ml. of bromobenzene (sp. gr. 1.49), technical grade, plus 150 ml. of kerosene (sp. gr. 0.80); white kerosene is preferable, although special purity is not required. If the specific gravity is not 1.07 ± 0.005 (urinometer), adjust to 1.07 with a little bromobenzene or kerosene.

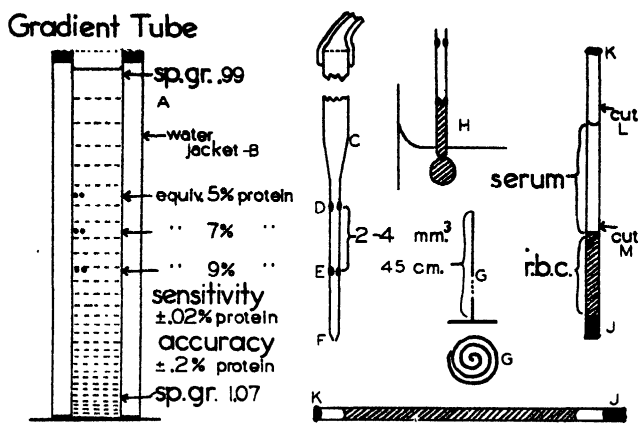


FIG. 1. Gradient tube for determination of serum protein concentration. *A*, graduated cylinder; *B*, water jacket; *C*, constriction pipette; *D*, *E*, and *F*, constrictions; *G*, copper wire stirrer; *H*, insertion of droplets; *J* and *K*, picene seals on blood-collecting tubes.

3. *Gradient Solution B*, 70 ml. of bromobenzene plus 180 ml. of kerosene. Adjust, if necessary, to a specific gravity of 0.99 ± 0.005.

4. *Jacket solution*, 0.2 per cent copper sulfate in 0.1 per cent sulfuric acid to fill the space between the two cylinders above. (The copper sulfate prevents molds and absorbs radiant heat.)

5. *Constriction pipettes* (Fig. 1, *C*), similar to those described by Linderstrøm-Lang (5) but adapted to deliver 2 droplets instead of 1. The constrictions differ in size, the upper one, *D*, being a little larger than the middle one, *E*, which is in turn larger than the bottom constriction, *F*. (These are obtainable from Eimer and Amend, New York, or may be constructed in the laboratory with a little practice, by the use of an injection needle (size 22 to 25) as a micro burner to make the constrictions.)

6. *Stirrer*, a wire spiral made from heavy copper wire, 12 to 16 gage (Fig. 1, *G*) (suggested by Dr. A. M. Butler).

7. *Blood-collecting tubes*, melting point capillaries, *H*, of 1.5 to 2.0 mm. outer diameter, 7 to 10 cm. long, open at both ends, for collecting blood (*e.g.* No. 34500 of the Kimble Glass Company, Vineland, New Jersey).

8. *Picene cement* for sealing the capillaries.[1]

9. *Fine sea sand* for removing droplets from the gradient tube.

10. *Specific gravity standards.*[2] 250 gm. of C.P. K_2SO_4 are dried overnight at 100–110°. 17.64, 23.08, 28.53, 34.04, 39.58, and 45.10 gm. amounts are weighed out (tolerance, 0.05 gm.) and each sample is diluted to 1 liter to give six standard solutions of specific gravity, 1.0141, 1.0184, 1.0227, 1.0270, 1.0313, and 1.0356, which are equivalent to serum protein concentrations of 2.5, 4.0, 5.5, 7.0, 8.5, and 10.0 per cent, respectively. These specific gravities are calculated *relative to water at the same temperature.* These standards will keep indefinitely if protected from mold and evaporation by storing in rubber-stoppered bottles in the ice box. For working standards, 5 or 10 ml. rubber-stoppered bottles or tubes are filled with these solutions. Every week or two the small vessels should be emptied and refilled from the large samples in the refrigerator.

Preparation of Gradient Tube—The graduated cylinder is filled to the middle graduation with *Solution A*, and on top of this *Solution B* is carefully layered to the upper graduation. This may be accomplished by filtering *Solution B* into the cylinder, arranging the funnel so that the solution runs down the cylinder wall. The space between the two cylinders is filled with the dilute acid copper sulfate.

The density gradient is now established by partially mixing the two layers with the copper spiral, *G*, in the following manner. Strokes of uniform velocity are made with the spiral between positions 3 or 4 cm. above and below the middle graduation until the schlieren effects (disturbances in transmitted light due to differences in refraction) are of nearly the same magnitude throughout the interval. The strokes are then lengthened by steps of 6 or 8 cm. until they reach the full length of the column. At each step, mixing is continued until the schlieren effects are of the same magnitude throughout the interval. It will perhaps be surprising to find how much stirring this requires. The usual mistake is to stir too little rather than too much. After 5 or 10 minutes, droplets of each of the standard solutions are put into the cylinder and the positions at which the droplets stop are noted. The total interval between the lightest and the heaviest standard should be one-half to one-fourth of the graduated length. The standard droplets should be nearly equally spaced (±20 per cent). If the droplets are too close together, more full length strokes should be made. If the gradient does not approximate linearity, it may

[1] Pyseal, Eimer and Amend, New York, or Plicene, Central Scientific Company, Boston.

[2] These may be obtained ready made from Eimer and Amend.

be brought into linearity by stirring more when the standards are too close together, though this will seldom be necessary. If the droplets are too widely spaced, there has been too much stirring. In this event the gradient should be reestablished as follows: The bromobenzene and kerosene are siphoned or poured out, completely mixed, and divided into two equal parts. Each part will now have a specific gravity of about 1.03. To one part are added 95 ml. of bromobenzene per liter to bring it to 1.07 specific gravity. To the other portion are added 210 ml. of kerosene per liter to bring it to a specific gravity of 0.99. With these as the starting solutions, the cylinder is filled as before and the gradient is reestablished as described above.

After the cylinder has stood for half an hour, the gradient is ready for use. It should be set on a level table out of direct sunlight and away from other sources of heat. A light background will facilitate reading. The gradient will improve with time, becoming more nearly linear. Eventually the intervals between standards will become greater and finally, after 6 months to a year, depending on use and protection from evaporation, the heaviest standard will fall through to the bottom. When this occurs, the gradient should be reestablished as described above. In warm weather the standards will all lie lower than in cold weather, since the temperature coefficient of water is much less than that of the organic solvents. If the gradient tube is not kept tightly stoppered when not in use, the more volatile bromobenzene will evaporate somewhat.

Use of Gradient Tube—The constriction pipette (Fig. 1) is rinsed two or three times and filled to the upper constriction with the heaviest standard, a rubber tube being used as with a blood-counting pipette. The pipette tip is gently wiped on a slightly moistened piece of filter paper or towel and introduced into the gradient tube on the extreme left side. With the tip just below the liquid surface and about 5 mm. from the wall, the solution is blown down to the middle constriction where surface tension stops it. By raising the tip of the pipette through the surface, the droplet pulls away and falls. The tip is put back into the liquid and the second droplet is delivered a few mm. behind the first. Care is taken not to blow air after the second droplet, as this would break it into a fine spray.

The rest of the standards are put directly on top of the first in the order of decreasing specific gravity. The pipette is rinsed several times with each new standard before use. For nearly normal sera the 5.5, 7.0, and 8.5 per cent protein standards will suffice. The positions of the standard droplets are read 4 minutes or more after the last standard has been introduced, by which time all will have reached equilibrium. It is more convenient and accurate to read the position of the lower edge of each droplet than to try to estimate the height of its center.

Serum is delivered in the same manner as the standards. Separate pipettes should be used for the standards and for serum, since serum leaves a film on the pipette which makes it difficult to pipette salt solutions. If there is insufficient serum available to rinse the pipette, it may be rinsed with water and dried with acetone before use. There is ordinarily little or no error if different sera are pipetted one after another without rinsing. If a pipette that has been used for serum remains idle for more than a moment, it should be rinsed with water, since otherwise the fine tip may dry shut. A dried tip can usually be reopened with concentrated nitric acid.

The serum droplets are delivered into the gradient tube a few mm. to the right of the standards. A duplicate droplet is unnecessary if there is a shortage of serum, but its presence increases confidence in the result and adds scarcely at all to the time consumed. Each serum is introduced in a separate "lane" a few mm. to the right of the previous one. Vertical marks at the top of the cylinder aid in placing the droplets in separate lanes. As many as ten or twenty serum samples may be inserted if care is taken to place them close to each other. If desired, a second row of droplets may be inserted 2 or 3 cm. behind the first. Each pair of droplets is read 4 ± 0.5 minutes after delivery. In the interval, the next three or four samples may be put in. With experience, no difficulty is encountered in keeping track of the time and order of the droplets. The positions of the standards are plotted on graph paper against their equivalent serum protein concentration, and from the resultant curve the unknown sera are evaluated. It is unnecessary to consider the temperature of the gradient tube at all, since both serum and standards have essentially the same temperature coefficient.

Removal of Droplets—After the column becomes full of droplets a little of the sea sand is sprinkled into the top of the gradient tube. The fine particles adhere to the droplets, carrying them to the bottom, and the gradient may be used again immediately.

Turbidity—If the gradient tube should ever become turbid (due to moisture), it may be clarified by grinding a little $CaCl_2$ with a few ml. of the top gradient mixture and quickly pouring the resultant suspension into the column. It will be necessary to wait some hours after this before using the gradient again, in order to give time for the finer particles of $CaCl_2$ to settle out.

Collection of Small Serum Samples—Since only a few c.mm. are required for serum protein determination with the gradient tube, it is possible to use the following simple method of serum collection. Capillary tubes, 1.5 to 2.0 mm. in outer diameter and 7 to 10 cm. long, are provided. Blood is taken from the finger or ear lobe, with care to wipe off the first droplet of blood and all traces of alcohol before taking the sample. Squeezing the

finger is permissible (see below). The capillary tube is about three-quarters filled by capillarity.[3] The tube is tipped until the blood runs to the middle, care being taken to keep one end of the capillary dry. The ends are sealed as follows: The stick of picene is softened in a flame (match, alcohol lamp, etc.) and applied to the *dry end*, J, of the capillary which has also been warmed in the flame. For the tight seal required at this end, the glass must be warm enough for the picene to "wet" it. The other end of the capillary, K, is capped with a little picene without warming the tube, which might otherwise produce hemolysis. The capillary is centrifuged 5 to 10 minutes at 3000 R.P.M. with the tightly sealed end down. A great number of samples may be centrifuged together by wrapping each tube in

TABLE I

Blood Samples Taken by Venipuncture and Capillary Puncture from One Individual within Short Space of Time

The values are given in gm. per cent. Each value represents a different capillary tube. The figures for finger and ear are given in the order in which they were taken.

Source of blood	Remarks	Serum protein	Source of blood	Remarks	Serum protein
Vein	Macro sample	6.76	Finger Puncture 2	Free flow	6.80
"	Centrifuged in capillary tubes	6.77		" "	6.63
		6.83		" "	6.65
		6.78		" "	6.66
Finger Puncture 1	Squeezing	6.72	Finger Puncture 3	Hard squeezing	6.76
	Slight squeezing	6.69		" "	6.76
	Free flow	6.65		" "	6.74
	" "	6.67	Ear	Free flow	6.72
	Squeezing	6.67		" "	6.76
				" "	6.76
				" "	6.76

a small piece of paper bearing identification. The protein should be measured within 8 hours if kept at room temperature, or within 48 hours if kept at 4–6°. When ready to make the determination, the top of the capillary above the serum is removed after scratching at L (Fig. 1) with a diamond point. A second scratch, M, is made just above the red cells and the serum segment is removed. The serum is drawn into the constriction pipette and transferred to the gradient tube as described above.

Venous Versus Capillary Blood—Table I demonstrates the correlation of blood samples taken from one individual by vein, finger, and ear. The

[3] This will furnish 4 or 5 times the amount of serum required for the protein determination; hence, if no other analyses are contemplated, a much smaller amount of blood may be taken.

venous samples were obtained from a large blood specimen, part of which was centrifuged as a large sample and part of which was transferred to capillary tubes for centrifugation. It would appear that centrifuging in capillary tubes does not change the specific gravity and that capillary blood and venous blood yield identical serum protein values within 0.1 gm. per cent. Furthermore, squeezing is without influence on the result obtained. This suggests that the well known influence of squeezing on hematocrit values is due to dilution with serum rather than to dilution with tissue extracellular fluid of lower protein concentration. Except perhaps in the case of Finger Puncture 2, the first sample agrees with the rest. Nevertheless, it would seem desirable in general to discard the first droplet as is done in taking blood for enumeration of red cells.

Effect of Storage—As with larger samples, the storage of whole blood in capillary tubes eventually changes the serum protein concentration (Table II). As might be anticipated, the changes occur more rapidly at room

TABLE II

Effect of Storage of Whole Blood in Capillary Tubes on Serum Protein Values

Temperature	Time stored				
	0 hr.	2 hrs.	6.5 hrs.	25 hrs.	48 hrs.
	Protein concentration, gm. per cent				
°C.					
30	6.83	6.93	6.90	7.30	7.52
4	6.83	6.89	6.88	6.87	6.91

temperature than at 4°. At the lower temperature there was little change in 48 hours. At 30° there was no significant change in 6 hours, but a definite increase in serum density occurred within 25 hours. This change is presumably the consequence of the swelling of red cells with attendant concentration of the serum, resulting from glycolysis.

Correlation with Kjeldahl Determination—In 240 individuals without obvious liver damage[4] there was found to be a standard deviation of 0.24 gm. per cent between serum protein values obtained with the gradient tube and those calculated from the total nitrogen (Kjeldahl). This correlation was obtained with the formula, per cent protein = 348 (sp. gr. 1.0069). This formula was employed since it gave slightly better correlation with the Kjeldahl values than either the formula of Moore and

[4] Cases with liver damage have been omitted because in many instances the gradient tube gave distinctly lower values (average 0.2 per cent) than were obtained by Kjeldahl determination. One of the authors (T. H. H.) will elaborate on this finding elsewhere.

Van Slyke (1) (protein = 343 (sp. gr. 1.007)), or that of Weech, Reeves, and Goettsch (2) (protein = 347.9 (sp. gr. 1.00726)). These latter formulae give serum protein values approximately 0.1 gm. per cent lower than the formula we have used.

Table III shows the correlation of serum protein concentrations obtained with the gradient tube with the values calculated from the total nitrogen. These 240 serum samples were obtained from patients with a variety of diseases. The protein concentrations varied from 3.5 to 13.5 gm. per cent. Many sera with abnormal albumin to globulin ratios are represented. It will be seen that only 5 per cent of the values obtained by the Kjeldahl procedure and the gradient tube deviate by more than ±0.4 gm. per cent. Looney (9) failed to obtain a high degree of correlation between serum specific gravity and serum protein concentration, but unfortunately his serum protein values are based on a turbidimetric procedure.

TABLE III

Correlation of Serum Protein Values Determined with Gradient Tube with Those Determined by Kjeldahl Analysis in 240 Normal and Pathological Sera

Deviation between methods, *gm. %*	±0.0	±0.1	±0.2	±0.3	±0.4	±0.5	±0.6	±0.7
% of determinations with given deviation	16	37	20	14	8	2	2	1
Cumulative % of determinations deviating more than this given deviation	84	47	27	13	5	3	1	0

DISCUSSION

From the foregoing it should be possible to assess the advantages and disadvantages of the gradient tube for the determination of serum protein. The advantages appear to lie in the smallness of the sample required, the speed of analysis, the freedom from influence of temperature or size of drop, and the lack of necessity for accurate timing. The $CuSO_4$ method (4) requires several hundred c.mm.,[5] the falling drop method 10 c.mm.,

[5] We have observed a discrepancy between the specific gravity measured by the gradient tube and by the copper sulfate method. For ten serum samples analyzed both ways the copper sulfate method gave specific gravity values *lighter* by an average of 0.0016 (standard deviation 0.0003). In comparison with the pycnometer the gradient tube appears to yield specific gravity values *too heavy* by about 0.0005, whereas in our hands the copper sulfate method gives values which appear to be *too light* by about 0.0010. Recently (10) a new formula has been introduced for use with the copper sulfate method which brings results by the two methods more nearly into agreement (per cent protein = 360 (sp. gr. 1.007) instead of per cent protein = 343 (sp. gr. 1.007)).

whereas for the gradient tube only 2 to 3 c.mm. are required. Indeed, if desirable, as little as 0.5 c.mm. in a smaller pipette will suffice.

Approximately one determination in duplicate per minute may be made with the gradient tube. This is somewhat faster than with any other specific gravity method. The freedom from dependence upon temperature, size of drop, and accurate timing is shared by both the gradient tube and $CuSO_4$ methods in contrast with the falling drop procedure. Perhaps these have been the chief disadvantages of the falling drop method.

The gradient tube shares in common with all other serum protein methods that are based on measurements of specific gravity the disadvantage that serum constituents other than protein influence serum density. Although this point will be discussed more fully in a subsequent paper, it may be noted that in order to influence the apparent serum protein concentration by as much as 0.1 gm. per cent it would be necessary to double the normal concentration of either the serum lipids, the blood glucose, or the non-protein nitrogen. Cholesterol is almost without effect, since its specific gravity is close to that of serum. With high serum lipid concentrations an error is encountered if the droplets are not read promptly at 4 minutes, since the droplets will fall further with time, presumably due to an absorption of bromobenzene. The data presented in this paper may be taken to indicate that it is rare for any of these factors to affect seriously the results obtained.

The gradient tube method has received extensive trial in the laboratories from which this report originates. To date approximately 5000 protein determinations have been made on sera, from clinical patients (2500), small laboratory animals (1000), and subjects of nutritional surveys (1500). The method has continued to give satisfaction throughout.

Use of Gradient Tube for Determination of Hemoglobin—The specific gravity of whole blood has been shown to be an accurate measure of its hemoglobin content, particularly if the serum specific gravity is also known (Phillips *et al.* (4)). The gradient tube has proved to be very convenient for this purpose and has been especially valuable in field studies when a colorimeter was not available. Only 4 or 5 c.mm. of blood are required for a duplicate determination. In the field, a cylinder (100 or 250 ml.) may be used without a water jacket, to save space. The only changes necessary are in the specific gravity range of the column and in the density standards. The lighter organic mixture is 69.5 volumes per cent kerosene, 30.5 per cent bromobenzene (sp. gr. 1.010); the heavier mixture is 55.0 per cent kerosene, 45.0 per cent bromobenzene (sp. gr. 1.110). The standards contain 57.13, 70.22, 83.49, and 96.86 gm. of K_2SO_4 per liter with a specific gravity of 1.0450, 1.0550, 1.0650, and 1.0750. When needed for very low hemoglobin values, the heaviest serum protein

standard (1.0356) may also be used. The gradient is produced and used exactly as described for serum protein. For simplicity, blood is drawn from the finger into a capillary tube (see above) having a small rubber bulb on one end. This rubber bulb is the type used for vaccination purposes and is perforated at both ends. The second perforation allows blood to enter the tube by capillarity, but on closure of this hole with the finger and squeezing the bulb, 2 droplets of blood may be delivered directly into the gradient tube. That the 2 droplets will seldom be of the same size is immaterial. The blood must, of course, flow freely from the finger with little or no squeezing. Calculation of the hemoglobin concentration is made from the nomogram of Phillips *et al.* (4).

SUMMARY

1. A density gradient tube suitable for the rapid determination of the specific gravity of 2 to 3 c.mm. of serum is described and directions given for its use.

2. The collection of a small amount of serum from the finger or ear for use in the gradient tube is described.

3. Comparison is made between serum protein concentration in serum from the vein, finger, and ear, and the influence of storage on the results obtained is shown.

4. In 240 individuals without liver damage serum protein values obtained with the gradient tube are correlated with those calculated from total nitrogen determinations. In individuals without liver damage, a standard deviation of 0.24 gm. per cent has been found between results by the two methods.

5. The use of the gradient tube for measuring blood hemoglobin concentration is described.

BIBLIOGRAPHY

1. Moore, N. S., and Van Slyke, D. D., *J. Clin. Invest.*, **8**, 337 (1930).
2. Weech, A. A., Reeves, E. B., and Goettsch, E., *J. Biol. Chem.*, **113**, 167 (1936).
3. Barbour, H. G., and Hamilton, W. F., *J. Biol. Chem.*, **69**, 625 (1926).
4. Phillips, R. A., Van Slyke, D. D., Dole, V. P., Emerson, K., Jr., Hamilton, P. B., and Archibald, R. M., *Bull. U. S. Army Med. Dept., No. 71*, 66-83 (1943).
5. Linderstrøm-Lang, K., and Lanz, H., Jr., *Compt.-rend. trav. Lab. Carlsberg, Série chim.*, **21**, 315 (1938).
6. Jacobsen, C. F., and Linderstrøm-Lang, K., *Acta physiol. Scand.*, **2**, 149 (1940).
7. Lowry, O. H., and Hastings, A. B., *J. Biol. Chem.*, **143**, 257 (1942).
8. Ponder, E., *J. Lab. and Clin. Med.*, **28**, 232 (1942).
9. Looney, J. M., *J. Lab. and Clin. Med.*, **27**, 1463 (1942).
10. Phillips, R. A., Van Slyke, D. D., Dole, V. P., Emerson, K., Jr., Hamilton, P. B., and Archibald, R. M., Copper sulfate method for measuring specific quantities of whole blood and plasma, Josiah Macy, Jr., Foundation, New York, February (1945).

THE POSITION OF FIXED CARBON IN GLUCOSE FROM RAT LIVER GLYCOGEN*

By HARLAND G. WOOD, NATHAN LIFSON, AND VICTOR LORBER

(From the Department of Physiology, University of Minnesota, Minneapolis)

(Received for publication, April 2, 1945)

The fixation of carbon dioxide in rat liver glycogen *in vivo* was first demonstrated by Solomon *et al.* (1). When radioactive sodium bicarbonate was injected intraperitoneally and lactate or glucose (2) was given by stomach tube to fasted rats, labeled carbon appeared in the liver glycogen.

Solomon *et al.* (1) proposed that the fixation Reaction 1 plays an important part in glycogen synthesis by providing a means for circumventing the only irreversible reaction in glycolysis; *i.e.*, the conversion of phosphopyruvic acid to pyruvic acid (3). The mechanism of this circumvention is represented empirically by the following steps:

$$CH_3 \cdot CO \cdot COOH + C^*O_2 \leftrightarrows C^*OOH \cdot CH_2 \cdot CO \cdot COOH \tag{1}$$

$$C^*OOH \cdot CH_2 \cdot CO \cdot COOH \leftrightarrows C^*OOH \cdot CH{:}CH \cdot COOH \tag{2}$$

$$C^*OOH \cdot CH{:}CH \cdot COOH \rightarrow CH_2{:}C(OPO_3H_2) \cdot C^*OOH + CO_2 \tag{3}$$

or

$$CH_2{:}C(OPO_3H_2) \cdot COOH + C^*O_2$$

The two types of phosphopyruvic acid of Reaction 3 with respect to labeled carbon (C*) are a consequence of the symmetrical nature of the dicarboxylic acid formed in Reaction 2. The remainder of the path to glycogen, Diagram 1, is viewed as the reversal of the current schemes of glycolysis involving combination of 2 molecules of phosphopyruvic acid, with an average of 1 out of 2 molecules possessing the fixed carbon as found in Reaction 3. The resulting glycogen should, therefore, contain 1 atom in 6 or 16.7 per cent of fixed carbon. By making certain assumptions, Solomon *et al.* (1) calculated for their experiments that the value of 16.7 per cent for fixed carbon in the glycogen was approached, but not significantly exceeded (except perhaps in one experiment in which the result was 20 per cent).

The current schemes for glycolysis provide (see Diagram 1) that the eventual combination of the 2 molecules of phosphopyruvic acid occurs at their carboxyl groups. If this is the case, the resulting 6-carbon chain should contain the fixed carbon entirely in positions 3 and 4, and the fixed carbon should, moreover, be equally distributed between these positions.

* Aided by grants from the National Foundation for Infantile Paralysis, Inc., and the Rockefeller Foundation, Research in Biology and Medicine.

The purpose of the present investigation has been, therefore, to determine whether or not the actual location of the fixed carbon in the glucose from liver glycogen is in agreement with these theoretical expectations.

Methods

The procedure as related to treatment of the animals was, with certain modifications, similar to that of Vennesland *et al.* (2). White rats weighing

DIAGRAM 1

Mechanism of Synthesis of Glycogen from Phosphopyruvate

$$\mathrm{CH_2{:}C(OPO_3H_2)\cdot C^*OOH} \rightleftharpoons \mathrm{CH_2OH\cdot CH(OPO_3H_2)\cdot C^*OOH}$$

Phosphopyruvate† 2-Phosphoglycerate

$$\rightleftharpoons \mathrm{CH_2(OPO_3H_2)\cdot CHOH\cdot C^*OOH} \rightleftharpoons \mathrm{CH_2(OPO_3H_2)\cdot CHOH\cdot C^*O(OPO_3H_2)}$$

3-Phosphoglycerate 1,3-Diphosphoglycerate

$$\rightleftharpoons \mathrm{CH_2(OPO_3H_2)\cdot CHOH\cdot C^*HO} \rightleftharpoons \mathrm{CH_2(OPO_3H_2)\cdot CO\cdot C^*H_2OH}$$

3-Phosphoglyceraldehyde Dihydroxyacetone phosphate

$$\rightleftharpoons \mathrm{CH_2(OPO_3H_2)\cdot COH\cdot C^*HOH\cdot C^*HOH\cdot CH\cdot CH_2(OPO_3H_2)}$$

Hexose diphosphate

$$\rightleftharpoons \mathrm{CH_2OH\cdot COH\cdot C^*HOH\cdot C^*HOH\cdot CH\cdot CH_2(OPO_3H_2)}$$

Fructose-6-phosphate

$$\rightleftharpoons \mathrm{CHOH\cdot CHOH\cdot C^*HOH\cdot C^*HOH\cdot CH\cdot CH_2(OPO_3H_2)}$$

Glucose-6-phosphate

$$\rightleftharpoons \mathrm{CH(OPO_3H_2)\cdot CHOH\cdot C^*HOH\cdot C^*HOH\cdot CH\cdot CH_2OH} \rightleftharpoons \text{glycogen}$$

Glucose-1-phosphate

† Phosphopyruvate is presumed to be provided by Reaction 3 in the text.

200 to 340 gm., fasted 48 hours, were fed by stomach tube 600 mg. of glucose per 100 gm. of body weight in either 20 or 30 per cent solution. The teeth of the animals were clipped prior to feeding. A total of about 10.0 ml. of 0.35 M $\mathrm{NaHC^{13}O_3}$ per 100 gm. of body weight was injected intraperitoneally in seven nearly equal portions at ½ hour intervals, the first injection occurring immediately after the glucose administration.

3½ hours after the feeding (½ hour after the last bicarbonate injection),

100 mg. of sodium amytal per 100 gm. of body weight were given intraperitoneally. Additional anesthetic was sometimes necessary because of the large amounts of fluid already present in the peritoneal cavity. When the anesthetic had taken effect, the peritoneal cavity was opened and the excess fluid contained therein was soaked up in weighed absorbent cotton sponges and held for analysis. The livers were extirpated and immediately placed in hot 30 per cent KOH. The glycogen was isolated and hydrolyzed to glucose.

In the later portions of the experimental period the rats appeared abnormally apathetic and their abdomens and flanks were markedly distended with fluid. The relatively massive doses of more than twice isotonic $NaHCO_3$ solution were used to achieve a sufficient heavy carbon content in the liver glycogen.

In two experiments the respiratory carbon dioxide was collected and its C^{13} content determined. The rat was placed in a wire mesh cage in a desiccator fitted with 2 N NaOH in an evaporating dish, calcium chloride, and a source of oxygen. The recovery of the total respiratory CO_2 was not quantitative, but was representative of the period of collection.

The position of the heavy carbon in the glucose was determined by two different types of degradation.

Bacterial Degradation of Glucose—The C^{13} glucose, which was isolated from the rat liver, was fermented to lactic acid with *Lactobacillus casei*. The lactate was then oxidized with $KMnO_4$ to acetaldehyde and CO_2; and the aldehyde was further degraded to iodoform and formic acid (4).

$$\text{(4)}\quad \underset{1}{\mathrm{CHOH}}\cdot\underset{2}{\mathrm{CHOH}}\cdot\underset{3}{\mathrm{C^*HOH}}\cdot\underset{4}{\mathrm{C^*HOH}}\cdot\underset{5}{\mathrm{CH}}\cdot\underset{6}{\mathrm{CH_2OH}} \rightarrow 2\mathrm{CH_3\cdot CHOH\cdot C^*OOH}$$

$$\underbrace{\qquad\qquad\qquad}_{\mathrm{O}}\ (1\text{–}5)$$

$$\text{(5)}\quad \mathrm{CH_3\cdot CHOH\cdot C^*OOH} \xrightarrow{\mathrm{KMnO_4}} \mathrm{CH_3\cdot CHO} + \underset{3,4}{\mathrm{C^*O_2}}$$

$$\text{(6)}\quad \mathrm{CH_3\cdot CHO} \xrightarrow{\mathrm{NaOI}} \underset{1,6}{\mathrm{CHI_3}} + \underset{2,5}{\mathrm{HCOOH}}$$

Chemical Degradation of Methyl Glucoside—The glucose was converted to methyl glucoside and oxidized with periodic acid at room temperature; carbon atom 3 is split out as formic acid (5) (first oxidation).

$$\text{(7)}\quad \mathrm{CH(OCH_3)\cdot CHOH.C^*HOH\cdot C^*HOH\cdot CH\cdot CH_2OH} \rightarrow$$

$$\underbrace{\qquad\qquad\qquad}_{\mathrm{O}}$$

$$\mathrm{CH(OCH_3)\cdot CHO\qquad C^*HO\cdot CH\cdot CH_2OH} + \underset{3}{\mathrm{HC^*OOH}}$$

$$\underbrace{\qquad\qquad\qquad}_{\mathrm{O}}$$

The formic acid was removed and recovered as CO_2 by oxidation with HgO (6), after which periodic acid was added to the remaining dialdehyde and the mixture was steam-distilled. At boiling temperature the glucosidic linkage of the dialdehyde is probably broken and the two resulting aldehydes are oxidized by the periodic acid (second oxidation). The principal products are formic acid, methyl alcohol, and formaldehyde, the latter arising from carbon atom 6, since it is known (7) that periodic acid converts the —CH_2OH groups of polyhydroxy compounds to formaldehyde.

$$\text{(8)} \qquad \underbrace{CH(OCH_3)\cdot CHO \qquad C^*HO\cdot CH}_{O}\cdot CH_2OH \rightarrow$$

$$CHO\cdot CHO + C^*HO\cdot CHOH\cdot CH_2OH + CH_3OH \rightarrow$$

$$\underset{1,2,5}{3HCOOH} + \underset{4}{HC^*OOH} + \underset{6}{HCHO} + CH_3OH$$

Details of the two methods are presented below.

Isolation of Glycogen—The glycogen isolation was carried out by the method of Good, Kramer, and Somogyi (8) with two alcoholic precipitations. The glycogen from each liver was hydrolyzed in 2 ml. of 1 N H_2SO_4 for 2½ to 3 hours in a boiling water bath, and determined as glucose-reducing equivalent by the Shaffer-Hartmann method. As indicated in Table I, the total carbon of the sample was grossly higher than that calculated from the glucose value; impurities were therefore present in the isolated glycogen.

Bacterial Degradation of Glucose—The fermentations were conducted with a suspension of *Lactobacillus casei*, which was centrifuged from a 3 day growth at 37° in glucose 1 per cent and Bacto-yeast extract 0.5 per cent. The cells were washed three times with 20 volumes of distilled water. The fermentation was carried out under CO_2 and at 37° in a Warburg respirometer with 150 ml. flasks. The reaction mixture contained 2 per cent wet bacteria, 0.06 M $NaHCO_3$, and the glycogen hydrolysate; the final volume was 30 ml. The sulfuric acid in the hydrolysate was previously neutralized with sodium hydroxide; the sulfate in the reaction mixture was thus less than 0.06 M. When the fermentation was complete, as judged by cessation of gas evolution, the bacteria were removed by centrifugation and the lactic acid was recovered by ether extraction.

The following procedure was used to remove miscellaneous impurities. A continuous 24 hour extraction with ethyl ether was made on the alkaline solution. This extract was discarded and the lactic acid was extracted from the acidified residue. The extract was then distilled with steam to remove volatile compounds. The lactic acid, which remained in the residue of distillation, was then oxidized with permanganate (9) and the acetaldehyde was collected in bisulfite, the carbon dioxide in alkali. The acetaldehyde

was further degraded by the iodoform reaction (4). The data are presented in Table II.

Preparation of Methyl Glucoside—The hydrolysate, containing 100 to 200 mg. of glucose, was neutralized to phenolphthalein with $Ba(OH)_2$ and was centrifuged, filtered, and evaporated to a thick syrup in a small beaker, in a vacuum desiccator at room temperature. The sugar was then transferred, with a total of about 5 ml. of water, to a 1.5 × 15 cm. Pyrex test-tube, and the drying continued. When completely dried, the material formed a brittle, foamy looking mass which was suitable for subsequent treatment.

Methylation was carried out in the test-tube according to the method of Fischer (10). To the dry residue, 2 ml. of anhydrous methanol-HCl (0.25 per cent HCl) were added and the mixture was refluxed for 30 to 45 minutes with frequent shaking. The test-tube was then sealed and placed in an oven at 100° for 50 hours. Following this, charcoal was added. The mixture was shaken briefly, permitted to stand for ½ hour, and was then filtered into a small weighed flask, with repeated methanol washing of the filter. Concentration to a thick syrup was carried out in an air bath at 60–70° under a current of air. The flask was seeded with a minute crystal of methyl glucoside and was rotated in an ice water bath while crystallization was taking place, permitting the formation of a thin film of crystals on the walls of the container. After standing in the ice box overnight, the crystals were covered with ethyl ether and permitted to stand, with one change of ether, for 24 hours. This treatment usually removed the faint yellowish discoloration from the crystals. The glucoside was dried in an oven at about 50° and weighed.

Yields by weight of about 75 per cent of theoretical were obtained by this method.

The copper-reducing power of the final product corresponded to a glucose content of 1 to 2 per cent, as judged by the result of control syntheses with pure glucose.

Chemical Degradation of Methyl Glucoside—The procedure used for the chemical degradation was as follows.

1 mM or less of solid methyl glucoside was oxidized for 2 hours at room temperature by dissolving it in 0.25 M HIO_4 (free acid, not acidified salt) equivalent to 2 mM of HIO_4 per millimole of glucoside.

This reaction mixture was diluted to 20 ml., 3 gm. of HgO were added, and the mixture was boiled and aerated for 20 minutes. The resulting CO_2 is from the formic acid of the first oxidation (carbon 3).

The mercury salts were removed by filtration into a steam distillation flask, and 25 ml. of 0.25 M HIO_4 were added; the total volume was now approximately 70 ml. Steam distillation was not begun until the volume was reduced to 15 ml. A total of 250 ml. of distillate was collected.

By simultaneous oxidation and steam distillation the formaldehyde was removed as it was formed; in this way secondary oxidation of the formaldehyde was reduced. Some CO_2 was formed during this step; its source in terms of glucose carbon atoms is unknown. This fraction is labeled CO_2 in the Tables III and IV.

20 gm. of HgO were added to the distillate, the mixture was boiled for 20 minutes, then 2 ml. of 1.7 M H_3PO_4 were added, and the mixture was boiled and aerated for 15 minutes. The resulting CO_2 is from the formic acid of the second oxidation.

The mercuric salts were removed by filtration. 25 ml. of 1 N NaOH were added to the filtrate, followed by 30 ml. of 0.05 N iodine. The ice-cold mixture was held for 10 minutes, then acidified with 0.1 ml. excess of 2 N H_2SO_4, and the excess I_2 was titrated with 0.1 N $Na_2S_2O_3$. The formaldehyde is converted to formic acid by this oxidation.

To the above solution 20 ml. of 0.3 M mercuric acetate and 5 ml. of 1 N acetic acid were added, and the mixture was boiled and aerated for 20 minutes (11). The resulting CO_2 is from the formic acid arising from the formaldehyde of the second oxidation (carbon 6). The conversion of the formaldehyde to formic acid with hypoiodite and to CO_2 with mercuric acetate was considered a reliable procedure for obtaining uncontaminated carbon from the formaldehyde.

The conversion of authentic formaldehyde to carbon dioxide by hypoiodite oxidation followed by mercuric acetate oxidation was found to be quantitative.

A number of other procedures for degradation of the methylated dialdehyde were attempted with less success, for example, hydrolysis with dilute hydrochloric acid followed by oxidation of the products with periodic acid. Refluxing with periodic acid, removal of the periodic acid as barium salt, and steam distillation of the products, formaldehyde and formic acid, were also tried. Bromine oxidation according to the procedure of Jackson and Hudson (5) was attempted without success, perhaps because of the limited amount of material available.

Heavy carbon was determined with the mass spectrometer. The C^{13} values are expressed as atoms per cent excess C^{13}; *i.e.*, per cent C^{13} in excess of the per cent of C^{13} in normal (unenriched) carbon. Carbon dioxide obtained from C.P. bicarbonate was assigned a value of 1.09 atoms per cent C^{13} and was used as a standard in calibrating the mass spectrometer. This calibration correction was not greater than ± 0.02 atom per cent. The per cents are calculated on the basis of the total carbon ($C^{12} + C^{13}$). The C^{13} contents of bicarbonate carbon, of normal animal glycogen carbon, and normal animal glycogen carbon isolated from 30 per cent KOH to which heavy bicarbonate was added all were found to be identical within ex-

perimental error. The error may be considered to be ±0.01 atom per cent C^{13}.

Results

Table I summarizes the general experimental data. It is evident that there was rapid transfer of the bicarbonate from the peritoneal cavity and that the glycogen hydrolysate of the liver contained excess C^{13} from the fixed carbon. The absorption of C^{13} bicarbonate was greater than the net absorption of bicarbonate, which indicates that there was an exchange of bicarbonate between the peritoneal contents and the body fluids.

TABLE I

General Experimental Data

Experiment No.	Rat weight	Bicarbonate administered intraperitoneally			Absorption from peritoneal cavity			Hydrolyzed glycogen		
		Volume	Molarity	C^{13}	Volume	HCO_3^-	C^{13}	Glucose	Total carbon as per cent of glucose value	C^{13} in total carbon
	gm.	*ml.*		*atoms per cent excess*	*ml.*	*per cent absorbed*	*per cent absorbed*	*mg.*		*atoms per cent excess*
I	200	21	0.35	5.1	−10.5	51	73	58	184	0.04
II	340	31.5	0.35	9.0	−8.7	51	79	65.5	154	0.07
	325	31.5	0.35	9.0	−8.7	54	79	89		
III	230	22.4	0.35	5.25				126	117	0.06
	240	22.4	0.35	5.25						
IV	200	21	0.35	5.25						
	270	28	0.35	5.25				287	137	0.038
	200	21	0.35	5.25						
V	225	16.8	0.135	5.25	+3.9	93	99	97		
VI	255	25.9	0.35	4.64	−11.8	61	85	122		

The results from the bacterial degradation of the C^{13} glucose from the rat liver glycogen are shown in Table II, in which it will be noted that all the excess labeled carbon appeared in the carboxyl group of the lactate.

The results from the chemical degradation of C^{13} methyl glucoside are shown in Table IV. Excess heavy carbon was present in the formic acid of the first and second oxidations, but there was none in the formaldehyde.

DISCUSSION

The conclusion we have drawn from these results is that *all the detectable fixed carbon is in positions 3 and 4 of the glucose chain.* The discussion presents the evidence and logic by which this conclusion is reached and a consideration of the inferences of this conclusion in relation to the biochemical significance.

Reliability of Bacterial Degradation of Glucose—There was reasonable agreement in the fermentation of the hydrolyzed glycogen between the millimoles of glucose (determined as reducing substance) and the lactic acid (determined by CO_2 evolution from bicarbonate). This fact indicates that the bacteria probably did not ferment non-carbohydrate impurities in the hydrolyzed glycogen. The aldehyde and CO_2 were formed in equal molar amounts in the permanganate oxidation of the residue of steam distillation, an indication that lactate was probably the only compound giving rise to these products. While the yield of iodoform and formic acid from the degradation of the acetaldehyde was only 55 per cent, this is similar to that obtained with known acetaldehyde. There is thus reason to believe that the bacterial degradation was specific for sugar carbon, and reliable.

The calculated values given in Table II for the degradation products were derived on the basis that all the excess C^{13} in the glucose was in positions 3 and 4; therefore the per cent excess C^{13} in these two positions is assumed to

TABLE II

Bacterial Degradation of C^{13} Glucose from Rat Liver Glycogen

The C^{13} values are in atoms per cent excess.

Fraction	Carbon atom of glucose	Experiment I C^{13}	Experiment II C^{13}
CO_2	3, 4	0.16*	0.28*
Formic	2, 5	−0.01	0.00
CHI_3	1, 6	0.00	−0.01

* The calculated values for the C^{13} in carbons 3 and 4 are 0.21 and 0.33 respectively in Experiments I and II.

be 3 times that of the whole glucose molecule. The C^{13} content of the glucose was calculated from the determined value for reducing sugars on the assumption that all the excess C^{13} was in the glucose and none was in the impurities of the hydrolyzed glycogen. In other words, the millimoles of excess C^{13} were considered to be the same in the glucose as that in the total carbon of the hydrolyzed glycogen.

The major limitation of both the bacterial and chemical degradations was that it was not possible to make a completely accurate quantitative estimate of the total proportion of labeled carbon in each degradation fraction. This limitation resulted from the error of the spectrometer (±0.01) and the relatively low C^{13} concentrations to be measured.

For example, if 10 per cent of the C^{13} of the total carbon in the hydrolyzed glycogen was in positions 2 and 5 of the glucose, the C^{13} content of this carbon would be raised to 0.02 per cent above the normal and the excess C^{13} would be just detectable by careful analysis.

With the exception of this limitation the results of the bacterial degradation are thought to be adequate and precise.

Reliability of Chemical Degradation of Methyl Glucoside—Two examples of results from the degradation of authentic methyl glucoside, which was synthesized from C.P. glucose (10), are shown in Table III. It is evident that there is some variation from the calculated values as based on Reactions 7 and 8.

The formic acid from the first oxidation was usually 100 per cent or greater than the calculated value. A low recovery, such as that shown in Experiment II of Table III, was the exception. The results of Jackson and Hudson (5) prove that it is carbon atom 3 that is split out in this oxidation and the present results indicate that there is 100 per cent cleavage.

TABLE III

Degradation of Authentic Methyl Glucoside

Fraction	Experiment I, Total carbon, Determined	Experiment I, Total carbon, Calculated*	Experiment II, Total carbon, Determined	Experiment II, Total carbon, Calculated*
	mM	mM	mM	mM
Methyl glucoside	4.95		4.91	
Formic acid, 1st oxidation (carbon 3)	0.77	0.71	0.64	0.70
" " 2nd "	2.21	2.83	2.48	2.81
Formaldehyde (carbon 6)	0.38	0.71	0.39	0.70
CO_2	0.41		0.55	

* Calculated on the basis that the degradation occurs according to Reactions 7 and 8.

The recovery of formaldehyde which is specific for carbon 6 (7) was somewhat more than 50 per cent.

The formic acid from the second oxidation is less well defined but is probably made up largely from carbon atoms 1, 2, 4, and 5 plus some of carbon 6. When 1.62 mM of authentic formaldehyde were subjected to the procedures involved in carrying out Reaction 8, there were obtained 0.33 mM of formic acid and 1.29 mM of formaldehyde. Apparently part of the formaldehyde was oxidized to formic acid. It, therefore, is to be expected that a part of the glucose carbon from position 6 will be included in the formic acid of the second oxidation. On a 4-carbon basis the recovery was approximately 80 per cent in the second oxidation. It is not known whether the formic acid arises in the same proportion from each of the 4 carbon atoms.

Methyl alcohol was not found to yield a significant quantity of formic

acid or formaldehyde when subjected to the degradation procedure. There is, therefore, no appreciable contamination from the alcohol which arises from hydrolysis of the glucoside.

Table IV contains analogous data for the chemical degradation of C^{13} methyl glucoside synthesized from glucose obtained from the experimental animals. For these samples the calculated millimoles of carbon in the various degradation fractions are made on the basis of Reactions 7 and 8 and the determined total carbon in the isolated methyl glucoside; *i.e.*, that the millimoles of methyl glucoside equal those of total carbon divided by 7.

Comparison of the yields of oxidation products from the isolated glucoside with that from known methyl glucoside reveals that there were discrepancies between the two degradations. The recovery of formic acid in the

TABLE IV

Chemical Degradation of C^{13} Methyl Glucoside from Rat Liver Glycogen

The C^{13} values are in atoms per cent excess.

Fraction	Experiment III				Experiment IV			
	Total carbon		C^{13}		Total carbon		C^{13}	
	Determined	Calculated	Determined	Calculated*	Determined	Calculated	Determined	Calculated*
	mM	*mM*			*mM*	*mM*		
Methyl glucoside	1.49			0.06	3.70		0.04	0.046
Formic acid, 1st oxidation (carbon 3)	0.20	0.21	0.15	0.21	0.43	0.53	0.10	0.156
Formic acid, 2nd oxidation	0.28	0.85	0.05	0.05	1.13	2.12	0.04	0.039
Formaldehyde (carbon 6)	0.08	0.21	0.00	0.00	0.31	0.53	0.00	0.000
CO_2	0.22		0.03		0.41		0.02	

* C^{13} of glucose (calculated as described in the text) × (6/7).

first oxidation and of formaldehyde was roughly in the same proportion as that from authentic methyl glucoside. The formic acid from the second oxidation was considerably lower. In Experiment III (Table IV), the deviation from the calculated millimoles is large; in Experiment IV, in which more material was used, the results were somewhat better. It is reasonably certain that part of these discrepancies are due to impurities in the methyl glucoside synthesized from rat liver glycogen. The actual amount of methyl glucoside probably was less than indicated by the total carbon.

For the purpose served, it is doubtful whether these impurities greatly impaired the reliability of the degradation. This view is taken, since in the bacterial fermentation substantially all the excess C^{13} of the hydrolyzed glycogen was accounted for fairly satisfactorily in the lactic acid. It is therefore unlikely that the non-carbohydrate impurities contained any ap-

preciable excess C^{13}. In this case inclusion of carbon from these impurities in a fraction of the degradation would only dilute the C^{13} in the particular degradation product of the glucoside. Such dilution would in part account for the fact that the C^{13} of the formate of the first oxidation (Table IV) was somewhat below the calculated value.

The principal use of the chemical degradation of glucose was to supplement the bacterial degradation and to locate more definitely the positions of the labeled carbon; *i.e.*, to determine whether the C^{13} was in the 1 or 3 and 4 or 6 carbon. Although the results (Table IV) may not possess the complete quantitative significance attributable to them had the glucoside been pure, they do justify the statements *that carbon atom 3 of the glucose contained labeled carbon (formic acid of first oxidation), that carbon atom 6 of the glucose did not contain labeled carbon (formaldehyde), and that there was labeled carbon in the glucose in some position or positions other than carbon atom 3 (formic acid of second oxidation).*

It has not been assumed, in determining the location of fixed carbon, that the bacterial fermentation occurs exactly as illustrated in Reaction 4. Only two assumptions have been made concerning the bacterial degradation; *i.e.*, that the lactic acid fermentation involves a cleavage of the 6-carbon glucose chain into two 3-carbon fragments which appear as lactic acid, and that there is no rearrangement of the carbon atoms in the chain. If this is the case, since excess C^{13} was present in only one position of the lactic acid, the carboxyl group (Table II), *not more than 1 carbon atom in each half of the glucose chain contained labeled carbon.* However, since *2* glucose carbons are included in the carboxyl group of the lactic acid, it cannot be ascertained by bacterial degradation alone to what extent the excess heavy carbon was present in one or both of these glucose carbons.

It further follows from the above assumption that *positions 2 and 5 of the glucose chain did not contain fixed carbon*, for the carbon atom of the carboxyl group of the lactic acid is the terminal atom of its 3-carbon chain and could have been derived only from positions 1 or 3 and 4 or 6 of the two halves of the glucose chain respectively.

These conclusions, when taken in conjunction with those of the chemical degradation, justify the further conclusion that *the labeled carbon is in positions 3 and 4 of the glucose chain.* There is no labeled carbon in positions 2 and 5 (bacterial degradation). The presence of labeled carbon in atom 3 (chemical degradation) excludes it from atom 1, since it cannot be in both atoms 1 and 3 (bacterial degradation). The absence of labeled carbon from atom 6 (chemical degradation) and the presence of labeled carbon in position 1, 2, 4, and 5, taken as a group (chemical degradation), require that labeled carbon is in position 4, since atoms 1, 2, and 5 have already been stated to be free of excess C^{13}. It may also be noted that, as nearly as can

be judged by the methods used, the fixed carbon is in approximately equal concentrations in positions 3 and 4.

Inferences and Biochemical Significance of Results—The question arises as to the significance of these results in interpreting the mechanism of fixation of carbon dioxide and of synthesis of glycogen.

It is apparent by examination of the equations in Diagram 1 that the results are in full agreement with the scheme proposed by Solomon *et al.* This result, of course, does not prove that the scheme is completely correct, but it does add considerable weight to the proposal. However, the same distribution of the labeled carbon would be obtained if labeled pyruvate was formed by a reversible equilibrium involving 2- and 1-carbon additions. Utter *et al.* (12) have demonstrated such a reaction with bacteria, but there is as yet no evidence of the reaction in animal tissue.

The present results do not answer the most important question concerning the scheme; *i.e.*, whether or not the fixation reaction is essential for the formation of phosphopyruvate. The Harvard investigators (1, 2, 13) attach significance to the observation that nearly the theoretical amount of glycogen as estimated from their scheme was found to be derived from carbon dioxide. This fact is taken to indicate that all the glycogen may be formed via the fixation reaction and that this was a consequence of the fact that the fixation mechanism was the only means of formation of phosphopyruvate from pyruvate. However, whether the fixation reaction is essential or non-essential, if it reaches equilibrium with the chain of reactions involved in glycogen formation, the concentration of fixed carbon will be the same in the glycogen.

There is some question concerning the maximum theoretical value for the concentration of fixed carbon dioxide in the glycogen. Actually, if the conversion of pyruvate to oxalacetate and fumarate (Reactions 1 and 2) was completely reversible and sufficiently rapid, both carboxyls of fumarate would come to equilibrium with the carbon dioxide. In this case the phosphopyruvate, as derived from the fumarate, would contain C^{13} in the carboxyl group in concentration equal to the free carbon dioxide at the place of reaction and the theoretical maximum would be 33.3 per cent rather than 16.7 per cent.

In the present investigation the theoretical value was not approached as closely as that attained by the Harvard investigators. In the experiments of the Harvard group the rats weighed 87 to 131 gm., were fasted for 24 hours, given intraperitoneally 7.5 to 8.4 ml. of a NaCl-$NaHCO_3$ solution, the bicarbonate concentration of which varied from a trace up to 0.126 M; the time of removal of the liver was 2½ hours after the first injection. In the present modification the rats were heavier, were fasted 48 hours, and

given intraperitoneally about 10 ml. of 0.35 M $NaHCO_3$ per 100 gm. of body weight, and the time of removal of the liver was 3½ hours. However, in a single experiment, Experiment V, Table V, we have eliminated some of these differences in details, the most important remaining discrepancy being perhaps in the weight of the animal. Table V shows a comparison between the results of Experiment V (Harvard method) and that of Experiment VI which may be taken as typical of the other experiments we have performed.

From Table V it would appear that in the Harvard method the atoms per cent excess C^{13} were not greatly different in the intraperitoneal fluid and respiratory air and presumably in the internal body fluids. In contrast, in Experiment VI, in which a large amount of bicarbonate was given, the atoms per cent excess C^{13} in the intraperitoneal fluid were considerably higher than in the blood or respiratory carbon dioxide, the amount in the latter two being not greatly different.

TABLE V

Comparison of Harvard Method and Method of Present Investigation

The C^{13} values are in atoms per cent excess.

	Experiment V, Harvard method C^{13}	Experiment VI, present method C^{13}
Carbons 3 and 4 of glucose	0.03	0.10
Respiratory CO_2, Sample 1	0.29	0.58
" " " 2	0.31	0.65
" " " 3	0.31	0.70
" " " 4		0.68
Intraperitoneal fluid	0.27	1.74
Blood bicarbonate		0.61

The C^{13} in carbons 3 and 4, which contained all the fixed carbon in the glucose, was found to have only one-tenth and one-seventh as much C^{13} excess in Experiments V and VI, respectively, as the respiratory carbon dioxide. Clearly the respiratory carbon dioxide did not approach equilibrium with carbons 3 and 4 of the glucose under the conditions of these experiments. The Harvard investigators report some experiments in which they obtained similar low fixation. It is possible that this small fixation may occur because part of the glucose is converted directly to glycogen without equilibration with phosphopyruvate. In this case the inclusion of carbon dioxide would not occur. In fact, Boxer and Stetten (14) have presented evidence that there is, in part, a direct conversion of glucose to glycogen which does not equilibrate with 3-carbon intermediates. It may be only under very limited conditions that complete equilibrium of glucose

and pyruvate is attained. Even when lactate is fed, it is conceivable that preformed glucose in the animal tissue may enter directly into the glycogen. However, Boxer and Stetten found the greater part of the glycogen molecule to arise from 3-carbon fractions under this condition. It seems likely that some approach other than determination of the amount of fixed carbon will be necessary to establish definitely whether or not the fixation reaction is essential for glycogen synthesis from pyruvate.

The present results give valuable information relative to the mechanism of the bacterial fermentation. Proof is provided that lactic acid is formed from the glucose carbon chain in such a way as to yield the same carbon distribution (Reaction 4) as is predicted for animal glycolysis. It does not necessarily follow that the intermediate mechanisms are identical, though this is probably the case (15). The bacterial fermentation provides a reliable method for degradation of glucose, which should prove valuable for further investigations.

It is to be noted that in neither the animal nor bacterial conversions was the labeled carbon randomized between positions 1 and 3, or 4 and 6 of the glucose. This fact proves that at no point in glycolysis is there a symmetrical 3-carbon intermediate. If dihydroxyacetone or a diphosphate ester of dihydroxyacetone was an intermediate, the labeled carbons of positions 3 and 4 of the glucose would have been converted into both methyl and carboxyl groups of the lactate. The present results are, therefore, in complete agreement with current concepts of the mechanism of glycolysis.

SUMMARY

The glycogen of rat liver was isolated following intraperitoneal administration of heavy carbon bicarbonate and feeding glucose by stomach tube. The position of the labeled carbon in the glucose from the glycogen was determined by bacterial and chemical degradation. The results show that carbon dioxide carbon is fixed in positions 3 and 4 of the glucose. The distribution of the isotope between the two positions is not indicated unequivocally by the data, but is tentatively considered to be equal. These observations are in accord with, but do not necessarily prove, the mechanism of glycogen synthesis as proposed by Solomon *et al.* (1). The C^{13} distribution in the glucose and in the lactic acid obtained by bacterial degradation of this glucose is that predicted by current concepts of glycolysis.

We wish to express our thanks to the staff in the Physics Department of the University of Minnesota, and especially to Dr. A. O. Nier, for their generous cooperation in this investigation, and to Miss Carol Carlson, who rendered valuable technical assistance.

BIBLIOGRAPHY

1. Solomon, A. K., Vennesland, B., Klemperer, F. W., Buchanan, J. M., and Hastings, A. B., *J. Biol. Chem.*, **140**, 171 (1941).
2. Vennesland, B., Solomon, A. K., Buchanan, J. M., and Hastings, A. B., *J. Biol. Chem.*, **142**, 379 (1942).
3. Meyerhof, O., Ohlmeyer, P., Gentner, W., and Maier-Leibnitz, W., *Biochem. Z.*, **298**, 396 (1938).
4. Wood, H. G., Werkman, C. H., Hemingway, A., and Nier, A. O., *Proc. Soc. Exp. Biol. and Med.*, **46**, 313 (1941).
5. Jackson, E. L., and Hudson, C. S., *J. Am. Chem. Soc.*, **59**, 994 (1937).
6. Osburn, O. L., Wood, H. G., and Werkman, C. H., *Ind. and Eng. Chem., Anal. Ed.*, **5**, 247 (1933).
7. Malaprade, L., *Bull. Soc. chim.*, **43**, 683 (1928); **1**, 833 (1934).
8. Good, C. A., Kramer, H., and Somogyi, M., *J. Biol. Chem.*, **100**, 485 (1933).
9. Wood, H. G., Werkman, C. H., Hemingway, A., and Nier, A. O., *J. Biol. Chem.*, **139**, 377 (1941).
10. Fischer, E., *Ber. chem. Ges.*, **28**, 1145 (1895).
11. Weike, H. D., and Jacobs, B., *Ind. and Eng. Chem., Anal. Ed.*, **8**, 44 (1936).
12. Utter, M. F., Werkman, C. H., and Lipmann, F., *J. Biol. Chem.*, **154**, 723 (1944).
13. Buchanan, J. M., Hastings, A. B., and Nesbett, F. B., *J. Biol. Chem.*, **145**, 715 (1942).
14. Boxer, G. E., and Stetten, D., Jr., *J. Biol. Chem.*, **155**, 237 (1944).
15. Werkman, C. H., and Wood, H. G., *Bot. Rev.*, **8**, 1 (1942).

THE ADENOSINETRIPHOSPHATASE ACTIVITY OF MYOSIN PREPARATIONS

By H. O. SINGHER* and A. MEISTER

(*From the Department of Physical Chemistry, Harvard Medical School, Boston*)

(Received for publication, March 19, 1945)

The recent finding that myosin preparations are capable of hydrolyzing adenosine triphosphate (ATP) has been considered evidence of a relationship between this protein and the energy-providing mechanisms of skeletal muscle. It has been reported by Engelhardt and Ljubimova (1, 2) that myosin prepared from rabbit muscle and precipitated by dilution, dialysis, salting-out, or acidification exhibited adenosinetriphosphatase activity. After three precipitations the preparations were capable of hydrolyzing one-half of the labile phosphorus of ATP and were incapable of acting upon adenosine diphosphate (ADP). Bailey (3) and others (4–7) have reported essentially similar results with rabbit myosin and myosin of other species. Thus far it has not been possible to separate the adenosinetriphosphatase activity from myosin and the tentative assumption has been made by some that the enzyme is, in fact, identical with myosin. Engelhardt *et al.* (8) have determined the effect of ATP upon myosin threads and have reported that these fibers increase in length when immersed in solutions of ATP. This is advanced as further evidence of a close relationship between myosin and ATP.

The obvious importance of these findings relative to muscular contraction initiated the series of experiments reported here. The present work, which differs in certain respects from that previously reported, deals with the hydrolysis of ATP by myosin preparations and the effects of various agents upon this reaction.

EXPERIMENTAL

Analytical Procedures—Phosphorus was determined by the method of Fiske and Subbarow (9). Nitrogen determinations were carried out by the procedure of Koch and McMeekin (10). The portable apparatus of Edsall and Mehl (11) was employed for observation of double refraction of flow.

Preparation of Adenosine Triphosphate—The calcium salt of ATP was prepared by the method of Fiske and Subbarow (12) and by that of Subbarow.[1] The barium salt was prepared according to the procedure of Kerr

* From part of the thesis presented by one of the authors (H. O. S.) to the Graduate School of Harvard University in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

[1] Subbarow, Y., unpublished.

(13). The sodium salt was prepared from the barium compound by precipitation with the stoichiometric amount of sulfuric acid. The solution was adjusted to pH 7 with sodium hydroxide, as determined by the glass electrode. The nitrogen to phosphorus and the 15 minute labile phosphorus[2] (which corresponds to the splitting of two phosphate groups from ATP) to total phosphorus ratios (atomic) for the preparations of ATP used in these experiments are as follows:

ATP preparation	N:P ratio	Ratio of labile to total P
Ca salt	5.04:3	2.0:3
" "	5.0:3	1.99:3
Ba "	5.03:3	2.03:3

Preparation of Myosin—Myosin was prepared by the method of Greenstein and Edsall (14) with several modifications. The muscles of the fore and hind limbs and the back of the rabbit were used. All procedures were carried out below 5°. The muscle was frozen with dry ice[3] and ground in a meat grinder to a fine powder. The powdered muscle was extracted with 10 times its volume of 0.5 M KCl and 0.04 M $KHCO_3$ buffer (pH 7.8, total molarity 0.54) with stirring for 16 hours. A second extraction was carried out on the muscle pulp with a smaller volume of buffer. The tissue was removed by high speed centrifugation. After combination of the extracts, the myosin was precipitated by dialysis against 0.005 M veronal-HCl buffer of appropriate pH. The precipitates were washed with the same solution and redissolved in veronal-HCl buffers brought to a salt concentration of 0.44 M by the addition of potassium chloride. The insoluble protein was removed by centrifugation. This solution will be referred to as Myosin 1. Three such precipitations were carried out. The redissolved material from each precipitation is labeled with the appropriate number. Preparations were made at pH values of 6, 7, 7.5, 8.0, and 8.5.

Measurement of Enzyme Activity—All solutions were brought to 25° before use. 2 cc. of an aqueous solution of Ca ATP were added to 1 cc. of myosin in a 10 cc. volumetric flask and mixed immediately. At the end of a given period the reaction was stopped by the addition of 4 cc. of 5 per cent trichloroacetic acid. The mixture was then made to volume with water, filtered, and a suitable aliquot analyzed for phosphorus. Control experiments were performed with ATP and buffer alone, and with myosin alone. The results are expressed as per cent of the *total* ATP phosphorus split. The different periods of hydrolysis were selected to obtain incomplete hydrolysis except in those cases in which total hydrolysis was

[2] The labile phosphorus was determined by hydrolysis with N HCl at 100° for 15 minutes.

[3] This procedure has no deleterious effect on myosin, as is discussed elsewhere (15).

to be observed. Bailey's value for the nitrogen content of myosin, 16.6 per cent, is employed throughout (16). All hydrolysis experiments were carried out in the presence of 0.25 M veronal-HCl-KCl buffers at pH 8.5. The enzyme activity in veronal buffer did not differ appreciably from the activities in borate or bicarbonate buffers of the same pH.

Results

Properties of Preparation—The results of determinations of nitrogen, enzymatic activity, and double refraction of flow of the successively precipitated myosin solutions are given in Table I. The values for the myosins

TABLE I

Properties of Myosin Solutions Capable of Splitting Adenosine Triphosphate (ATP)

Myosin preparation No. (pH 8.5)	Protein per cc.	ATP per cc.*	Hydrolysis (30 min.)	Double refraction†
	mg.	*mg.*	*per cent*	
1	2.65	0.600	65	2.5
2	2.42	0.764	60	2.1
3	2.35	0.654	60	2.4

* All ATP or P concentrations represent mg. of the calcium salt, unless otherwise specified.

† Expressed in terms of $(n_e - n_o) \times 10^{-6}$, where n_e and n_o are the refractive indices of the components of the light perpendicular and parallel to the optic axis. These measurements were made at a velocity gradient of 91 $sec.^{-1}$.

prepared at pH values of 7, 7.5, and 8 were similar to those for the preparation at pH 8.5. The myosin prepared at pH 6 showed no enzymatic activity, but there was no corresponding loss of double refraction of flow. On the basis of the constancy of nitrogen, enzymatic activity, and double refraction in Myosin 2 and Myosin 3, it was believed that the limits of fractionation obtainable by the dialysis procedure were reached.

Effect of Myosin and ATP Concentrations—With ATP concentrations of 0.875 mg. per cc. and 30 minute hydrolyses, the activity was found to remain constant at protein concentrations greater than 1.45 mg. per cc. The activity fell off rapidly at lower protein concentrations. Enzymatic activity was proportional to protein concentration at pH 7 below 2.0 mg. per cc. (Fig. 1). The succeeding experiments were carried out with a protein concentration of 1.45 mg. per cc., except in certain cases noted.

The effect of varying the ATP concentration was determined by 30 minute hydrolyses. The per cent hydrolysis was found to fall off at concentrations of ATP higher than 0.944 mg. per cc. (Table II).

Effect of pH and Temperature—The pH-activity curve was determined by

15 and 3 minute hydrolyses. The protein concentration was 1.2 mg. per cc. and the ATP concentration was 0.873 mg. per cc. The optimum pH

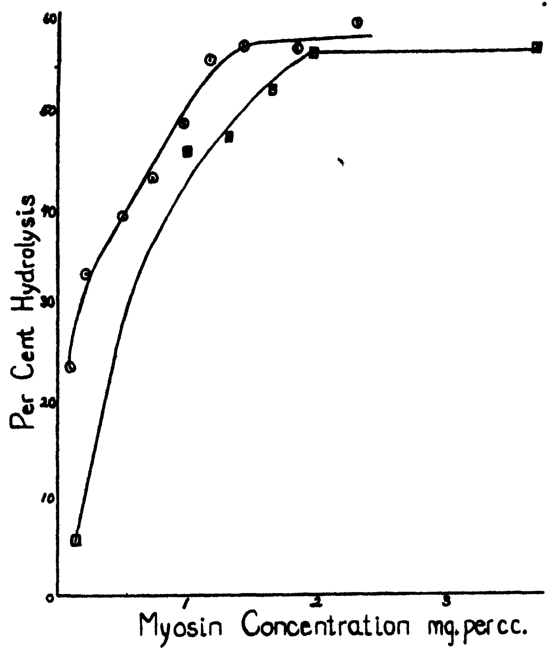


FIG. 1. The effect of myosin concentration on the splitting of adenosine triphosphate. □ 3 minute hydrolysis, ○ 15 minute hydrolysis.

TABLE II

Effect of Substrate Concentration on Splitting of Adenosine Triphosphate (ATP)

pH 8.0, 25°, time of hydrolysis 30 minutes, protein concentration 0.24 mg. of N per cc.

ATP per cc.	Hydrolysis	ATP per cc.	Hydrolysis
mg.	*per cent*	*mg.*	*per cent*
0.235	49.5	0.944	49.7
0.393	51.0	1.39	38.7
0.589	50.0	9.64	24.2
0.856	48.4		
0.883	49.6		

Note that complete splitting of *two* phosphate groups from ATP would be denoted as 66.7 per cent hydrolysis by our definition.

was found to be in the neighborhood of pH 9 in both sets of hydrolyses (Fig. 2). It was found that myosin solutions brought to values higher than pH 9.8 or lower than pH 6.0 by addition of dilute sodium hydroxide or dilute

hydrochloric acid, respectively, showed either diminished activity or complete loss of activity when the pH was readjusted to 8.5.

The optimum temperature was determined by 30 minute hydrolyses with 0.971 mg. of ATP and 1.45 mg. of protein per cc. The optimum temperature under these conditions was found to be in the neighborhood of 38° (Fig. 3).

Rate of Reaction—The rate of reaction was determined with the same concentrations of enzyme and substrate which were employed for the optimum temperature experiments. One-third of the total ATP phosphorus is liberated in 5 minutes, and the reaction goes virtually to completion,

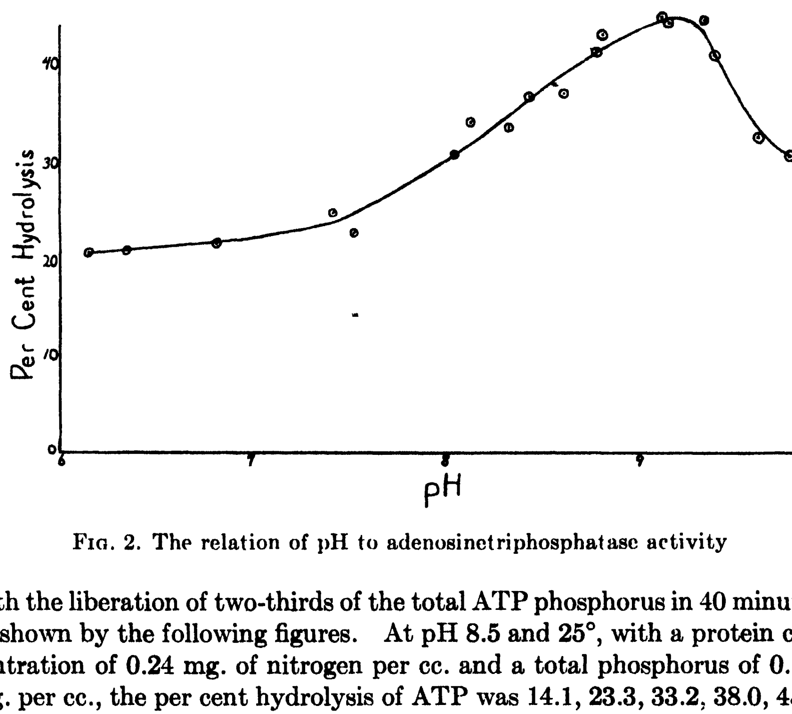


FIG. 2. The relation of pH to adenosinetriphosphatase activity

with the liberation of two-thirds of the total ATP phosphorus in 40 minutes, as shown by the following figures. At pH 8.5 and 25°, with a protein concentration of 0.24 mg. of nitrogen per cc. and a total phosphorus of 0.178 mg. per cc., the per cent hydrolysis of ATP was 14.1, 23.3, 33.2, 38.0, 45.1, 49.6, 59.8, 66.4, and 66.4 at 1.5, 3, 5, 10, 15, 20, 30, 40, and 60 minutes, respectively.

Effect of Activation—Enzymatic hydrolyses were carried out with sodium ATP in the presence of varying concentrations of $CaCl_2$ and $MgSO_4$. These experiments were run for 15 minutes and the concentration of ATP was 0.437 mg. per cc. Calcium concentrations of about 10^{-3} M and magnesium concentrations of 10^{-4} and 10^{-5} M had an activating effect. Higher concentrations of these ions produced diminution of activity. Activation with $MgCl_2$ was similar to that observed with $MgSO_4$. The

concentration of ATP was 0.545 mg. per cc. in the experiments with glycine. 1 M glycine produced activation (Table III).

Effect of Inhibiting Agents—A sample of myosin containing 7.2 mg. of protein per cc. was incubated at 37.5° and samples were withdrawn at intervals for determination of enzyme activity and the presence of double refraction of flow. Enzymatic hydrolyses were carried out for 15 minutes on 1 cc. samples, with 1.09 mg. of Ca ATP per cc. The activity decreased

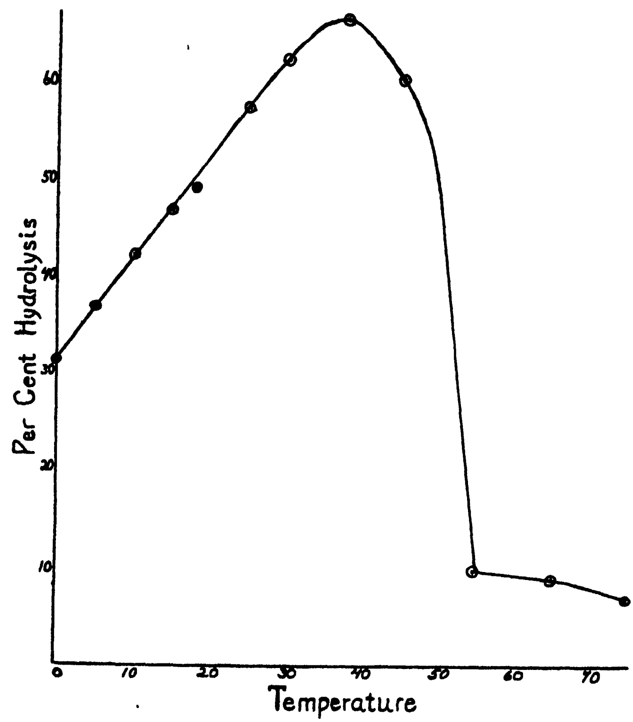


FIG. 3. The effect of temperature on the hydrolysis of adenosine triphosphate by myosin.

after 45 minutes of incubation, but showed no further diminution after 6 hours of incubation. Double refraction of flow was absent after 4½ hours of incubation. The effect of incubation at 37.5°, pH 8.5, on ATP splitting is shown by hydrolysis of 45.0, 35.6, 34.8, 32.6, 32.5, 32.6, 32.6, 32.5, and 34.7 per cent for 0, 45, 90, 180, 210, 240, 270, 300, and 360 minutes of incubation, respectively.

Myosin was treated with 0.8 M guanidine hydrochloride. Double refraction of flow and enzyme activity were found to be absent in this solution

and in the solution of myosin washed free of guanidine hydrochloride and redissolved in KCl solution. Similar results were obtained with 0.6 M $CaCl_2$. A partial loss of both properties was observed after treatment with 1.14 M potassium fluoride.

15 minute hydrolyses were carried out in the presence of varying concentrations of KCl and an ATP concentration of 0.71 mg. per cc. The amount of hydrolyzed phosphorus remained constant at all KCl concentrations below 0.7 M, but decreased at higher salt concentrations. At pH 8.5 and 25°, with a total P of 0.13 mg. per cc. and a protein N of 6.24 mg. per cc., the per cent hydrolysis of ATP in 15 minutes was 46.8, 46.5, 46.6, 45.0,

TABLE III

Effect of Activators on Splitting of Adenosine Triphosphate

Activator	Molarity	Hydrolysis
		per cent
None		46.6
Mg	0.00001	66.8
"	0.0001	52.5
"	0.006	25.1
"	0.03	23.9
"	0.3	16.1
Ca	0.0009	60.9
"	0.004	60.2
"	0.04	52.0
"	0.1	41.9
"	0.2	34.1
Glycine	1.0	59.5
"	2.0	48.4

39.2, 31.8, 29.8, 17.3, and 12.8 for 0.19, 0.25, 0.38, 0.69, 0.86, 1.53, 1.86, 2.5, and 3.5 M KCl, respectively. The same results were obtained with myosin prepared at different pH values.

DISCUSSION

The present results indicate that myosin prepared at pH 6 possesses no adenosinetriphosphatase activity, or in any case that the activity is rapidly lost when myosin is exposed to this pH. Bailey's myosin prepared at pH 6.8 to 7.0 split ATP but not ADP. On the other hand, myosin prepared in alkaline media (pH 7 to 8.5) hydrolyzes both labile phosphate groups of ATP. Summerson and Meister (17) have recently found that myosin prepared by repeated precipitation at alkaline reaction retains the ability to hydrolyze completely both labile groups. They refer to this preparation as Myosin 2. Precipitation of myosin at pH 6.5 produced myosin preparations similar to those of Bailey (Myosin 1).

Kalckar has recently suggested that the dephosphorylation of ATP in muscle may be due solely to myosin and myokinase (18). Colowick and Kalckar had previously shown that myokinase catalyzes the transfer of phosphorus from 1 molecule of ADP to another, producing, from 2 moles of ADP, 1 mole each of ATP and adenylic acid (19). It is quite possible that the ability of Myosin 2 to act upon ADP is due to the presence of myokinase in these preparations. If this is true, it would appear that myokinase is more readily separable from myosin at pH 6.5 to 7.0 than at pH 7 to 8.5.

The striking activation by magnesium of myosin prepared in alkaline media is not observed with Myosin 1. Bailey reports slight activation by magnesium of the thrice precipitated myosin in contrast to the marked activation obtained with once precipitated myosin. Magnesium augments the activity of myokinase considerably (19). The results of the studies on the effect of magnesium on the activity of Myosins 1 and 2 are, therefore, compatible with the suggestion that myokinase is responsible for the apparent hydrolysis of ADP by Myosin 2.

Our results confirm the previously reported data concerning pH and temperature optima (3) and glycine and calcium activation (3). The activation by calcium noted by us could be due solely to its effect on the Myosin 1-ATP reaction or on both components of Myosin 2. However, we have been unable to find any mention in the literature concerning the effect of calcium on myokinase.

In general the experiments reported here were carried out with high protein and low substrate concentrations. Hence, any calculation of activity similar to Bailey's Q_p values would be of little significance.

Our results disagree with those of Engelhardt and Ljubimova (1, 2) who report complete destruction of adenosinetriphosphatase activity of myosin by incubation for 15 minutes at 37°. We find considerable activity remaining after 6 hours of incubation at this temperature. We believe that the concentration of myosin was much lower in the cited experiments than in the present series. This might well explain the more rapid loss of activity in Engelhardt and Ljubimova's experiments, since enzymes and other proteins are frequently unstable in very dilute solutions.

A decrease in enzyme activity has also been observed by Mehl and Sexton (20) in rat myosin preparations with increasing concentrations of salt (0.1 to 0.5 M). In our experiments no decrease of activity was noted over the same range of salt concentration. An increase of the molarity of potassium chloride to 0.86, however, produced a diminution in activity. This apparent discrepancy is undoubtedly due to the different concentrations of myosin employed. Mehl and Sexton used myosin concentrations of 0.123 to 0.205 gm. per liter, while the myosin concentration in our experiments

was 1.45 gm. per liter. Apparently the effect of salt on activity is greater at lower protein concentrations. This has been found to be the case with other enzymes such as urease.

It has been shown that there is no correlation between the number of porphyrindin-titratable sulfhydryl groups of myosin and the presence of double refraction of flow (14). Guanidine hydrochloride (0.8 M), which increases the number of titratable sulfhydryl groups and destroys double refraction of flow, was observed to destroy the enzyme activity. Calcium chloride (0.6 M) has no effect on the number of sulfhydryl groups (as determined by the porphyrindin titration), but also destroys double refraction of flow and enzyme activity. Glycine, which reduces the titratable sulfhydryl groups to zero, has no effect on double refraction of flow, and enhances enzymatic activity. Barron and coworkers (21, 22) have reported inhibition of the myosin adenosinetriphosphatase by mercaptide-forming compounds and mild oxidizing agents, and reactivation of the enzyme with glutathione. Thus it appears that there may be, under some circumstances, a relationship between the sulfhydryl titer and enzyme activity. The lack of correlation between sulfhydryl content and double refraction of flow, however, makes it impossible to relate enzyme activity to double refraction.

Furthermore we have been able to prepare myosin which showed double refraction of flow (under our conditions) in the absence of enzymatic activity, and myosin which showed no double refraction of flow in the presence of adenosinetriphosphatase activity. The former myosin was obtained by precipitation of the protein at pH 6, while the latter was prepared by incubating the myosin at 37.5° (see "Effect of inhibiting agents"). In the second case no double refraction of flow was observed with a velocity gradient of 91 sec.$^{-1}$. We believe that double refraction of flow is a fair criterion of undenatured myosin. Engelhardt's data (8) also show that salting-out of the protein does not destroy enzymatic activity. Von Muralt and Edsall (23) and Edsall and Mehl (11) demonstrated that this procedure brings about a loss of double refraction of flow. It appears, therefore, that it is possible to separate the optical activity from the enzymatic activity. These results are apparently in disagreement with the view that myosin and adenosinetriphosphatase are identical. However, it is possible that both the double refraction of flow and the enzyme activities reside within a single myosin molecule and that the molecule may be decreased in length, producing loss of double refraction of flow (under the experimental conditions), without loss of enzyme activity. The other possibility which must be considered is that there exists in the myosin solution a heterogeneous protein population, as seems probable on the basis of work reported by Weber (24). The enzymatic activity might reside

in molecules that do not exhibit double refraction of flow at low velocity gradients.

The importance of the identity or close association of adenosinetriphosphatase activity and myosin becomes apparent with the work of Engelhardt on the change in length of myosin fibers in ATP solutions and that of Dainty *et al.* on the effect of ATP on myosin solutions (25). Engelhardt's experiments suggest a possible connection between the chemical and mechanical events occurring in muscular contraction. The present suggestion as to the non-identity of the birefringent and enzymatic properties of myosin would seem to still leave the question of the transformation of chemical to mechanical energy open to other possible explanations.

It is a pleasure to express our thanks to Dr. J. T. Edsall and Dr. E. J. Cohn for their interest and criticism during the course of this work.

SUMMARY

1. Myosin prepared in alkaline media possesses adenosinetriphosphatase and adenosinediphosphatase activity and shows double refraction of flow. Myosin prepared at pH 6 possesses neither of the adenosinephosphatase activities but shows double refraction of flow.

2. The effects of myosin concentration, substrate concentration, pH, temperature, and calcium, magnesium, guanidine hydrochloride, glycine, and potassium chloride concentrations were studied. Striking activation of the enzymatic activity of the myosin prepared in alkaline media was observed with calcium and magnesium.

3. Incubation of myosin at 37.5° produced slight reduction in enzymatic activity and complete loss of double refraction of flow under our conditions.

BIBLIOGRAPHY

1. Engelhardt, V. A., and Ljubimova, M. H., *Nature*, **144**, 668 (1939).
2. Ljubimova, M. H., and Engelhardt, V. A., *Biokhimiya*, **4**, 716 (1939).
3. Bailey, K., *Biochem. J.*, **36**, 121 (1942).
4. Ljubimova, M. H., and Pevsner, D., *Biokhimiya*, **6**, 178 (1941).
5. Szent-Györgyi, A., and Banga, I., *Science*, **93**, 158 (1941).
6. Sakov, N. E., *Biokhimiya*, **6**, 163 (1941).
7. Needham, D. M., *Biochem. J.*, **36**, 113 (1942).
8. Engelhardt, V. A., Ljubimova, M. H., and Meitina, R. A., *Compt. rend. Acad. sc. U. R. S. S.*, **30**, 644 (1941).
9. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
10. Koch, F. C., and McMeekin, T. L., *J. Am. Chem. Soc.*, **24**, 2066 (1924).
11. Edsall, J. T., and Mehl, J. W., *J. Biol. Chem.*, **133**, 409 (1940).
12. Fiske, C. H., and Subbarow, Y., *Science*, **70**, 381 (1929).
13. Kerr, S. E., *J. Biol. Chem.*, **139**, 121 (1941).
14. Greenstein, J. P., and Edsall, J. T., *J. Biol. Chem.*, **133**, 397 (1940).

15. Singher, H. O., Dissertation, Harvard University (1942).
16. Bailey, K., *Biochem. J.*, **31**, 1406 (1937).
17. Summerson, W. H., and Meister, A., Abstracts, American Chemical Society, New York, 42B (1944).
18. Kalckar, H. M., *J. Biol. Chem.*, **153**, 355 (1944).
19. Kalckar, H. M., *J. Biol. Chem.*, **148**, 127 (1943).
20. Mehl, J. W., and Sexton, E. L., *Proc. Soc. Exp. Biol. and Med.*, **52**, 38 (1943).
21. Barron, E. S. G., Singer, T. P., and Schramm, G., *Science*, **97**, 356 (1943).
22. Singer, T. P., and Barron, E. S. G., *Proc. Soc. Exp. Biol. and Med.*, **56**, 120 (1944).
23. von Muralt, A. L., and Edsall, J. T., *J. Biol. Chem.*, **89**, 315, 351 (1930).
24. Weber, H. H., *Kolloid-Z.*, **100**, 242 (1942).
25. Dainty, M., Kleinzeller, A., Lawrence, A. S. C., Miall, M., Needham, J., Needham, D. M., and Shen, S. J., *J. Gen. Physiol.*, **27**, 355 (1944).

CARBOHYDRATE CHARACTERIZATION

V. ANHYDRIZATION OF THE ALDOPENTO-BENZIMIDAZOLES*

By CHARLES FERDINAND HUEBNER, ROLLAND LOHMAR, ROBERT J. DIMLER, STANFORD MOORE, AND KARL PAUL LINK

(From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison)

(Received for publication, March 19, 1945)

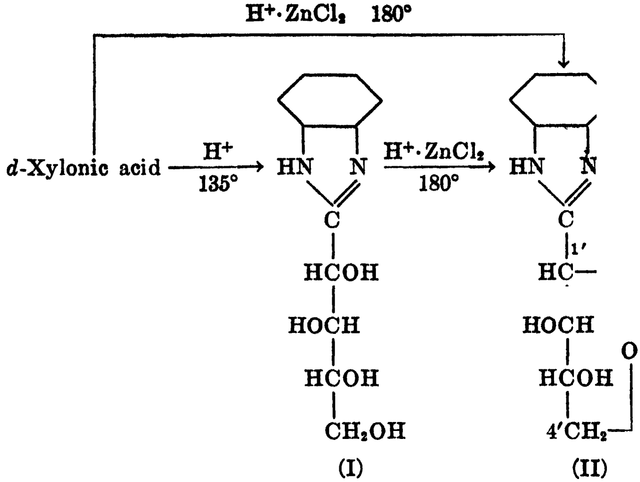
In communications (1, 2) dealing with the characterization of aldoses as benzimidazole derivatives, it was reported that *d*-xylonic acid reacts with *o*-phenylenediamine at 135° in the presence of acid to form a derivative (I) which is unique among the 2-(aldo-polyhydroxyalkyl)benzimidazoles[1] in being very soluble in water. It was further reported that condensation at 180° in the presence of zinc chloride and acid yields a water-insoluble product (II). The nitrogen analyses (Dumas) reported indicated that the product formed at 180° was *d*-xylo-benzimidazole, $C_{11}H_{14}O_4N_2$, and that the product obtained at 135° had the formula $C_{11}H_{16}O_5N_2$, indicating a compound which might have been formed by the elimination of 1 mole of water, instead of 2, from the reactants. Reexamination of these products showed the analyses to be in error because of the difficulties of complete combustion in the Dumas method. This characteristic is shared by other benzimidazole derivatives, particularly those of the saccharic acid series. Kjeldahl nitrogen values with the Elek and Sobotka modification (3) and carbon and hydrogen values now show the product formed at 135° to be *d*-xylo-benzimidazole (I), $C_{11}H_{14}O_4N_2$, whereas the product formed at 180° is an anhydro-*d*-xylo-benzimidazole (II), $C_{11}H_{12}O_3N_2$.[2]

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station, and supported in part through special grants from the Graduate Research Committee of the University, Office of Professor E. B. Hart, made available by the Wisconsin Alumni Research Foundation. Part of this work was presented at the New York meeting of the American Chemical Society, Division of Sugar Chemistry and Technology, September 15, 1944.

We are greatly indebted to Hoffmann-La Roche, Inc., Nutley, New Jersey (through Dr. H. M. Wuest), for a generous gift of *d*-ribose and to the Abbott Laboratories, North Chicago, Illinois (through Dr. E. H. Volwiler), for a special grant to help defray the cost of this study.

[1] For convenience these derivatives are given the trivial name, aldo-benzimidazoles. Individual members are named for the aldose from which they are derived; *i.e.*, *d*-gluco-benzimidazole.

[2] Professor C. S. Hudson, National Institute of Health, Bethesda, Maryland, also prepared II and kindly informed us, in a private communication, September 1, 1942, that his analyses agree with ours.



The proof of structure II is presented here. II contains a furane ring and is 2-(1′,4′-anhydro-*d*-xylo-tetrahydroxybutyl)benzimidazole. This conversion of the aldopento-benzimidazoles to their 1′,4′-anhydrides at 180° has been extended to arabinose, lyxose, and ribose.[3] In a subsequent paper, it will be shown that an inversion at carbon atom 1′ does not occur during anhydrization and that the original configuration of the aldopento-benzimidazole is retained in the anhydro derivative.

Assignment of the *d*-xylo-benzimidazole structure to I rests on the following evidence. Oxidative degradation of I with permanganate yielded 2-benzimidazolecarboxylic acid (V). Further support for the presence of a benzimidazole structure in the molecule is given by ultraviolet absorption spectra. I has an absorption curve with well defined maxima and minima identical with that of *d*-gluco-benzimidazole (Table I). The aldopento-benzimidazoles (as typified by *d*-arabo-benzimidazole) consume 3 molar equivalents of periodate to produce 2-benzimidazolealdehyde, formaldehyde, and 2 molar equivalents of formic acid.

The following considerations lead to the designation of II as 2-(1′,4′-anhydro-*d*-xylo-tetrahydroxybutyl)benzimidazole. Tests for a ketone

[3] The 180° transformation product from *d*-ribo-benzimidazole is extremely soluble in water and difficult to crystallize. The analyses of this low melting product (82–83°) establish its empirical formula as $C_{11}H_{12}O_3N_2 \cdot 2H_2O$. The water of hydration is not lost on drying *in vacuo* at 65°. It appears, however, that this compound is a hydrate of anhydro-*d*-ribo-benzimidazole, rather than a product in which the 2 water molecules have entered into the ultimate molecular constitution, because it undergoes the two reactions characteristic of the anhydro-aldopento-benzimidazoles; *viz.*, the formation of 2-(α-furyl)benzimidazole on treatment with acetic anhydride and the production of 2-benzimidazolecarboxylic acid on periodate oxidation.

group with phenylhydrazine, for an enolic group with diazomethane, and a lactol hydroxyl group with methanolic hydrogen chloride were all negative, thereby excluding possible $C_{11}H_{12}O_3N_2$ structures containing these groups. Since II could be converted to N-benzyl-anhydro-*d*-xylo-benzimidazole, it was concluded that a free imino group is present in II. The anhydro-*d*-arabo-benzimidazole in which *cis* hydroxyls are present on carbon atoms 2′ and 3′ formed an acetone condensation product. Since this compound could not be acetylated, the parent anhydro-benzimidazole contains but two hydroxyls. This compound apparently does not undergo N acylation; hence the remaining two hydroxyls originally present in *d*-arabo-benzimidazole must be engaged in ether linkage in the anhydro derivative. Because of the stability of the anhydro ring to reagents like sodium methoxide and strong acid, a furane type of ring was considered most probable.

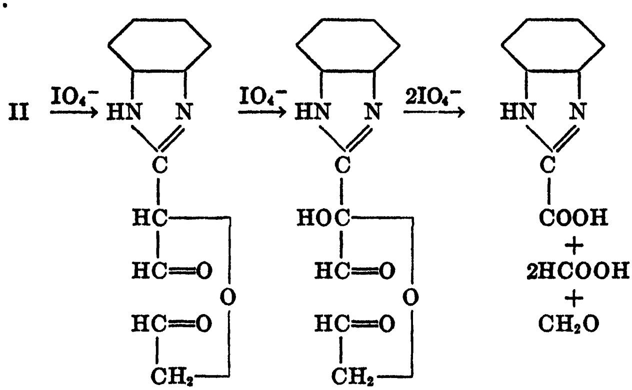
TABLE I

Benzimidazole Ultraviolet Absorption Spectra

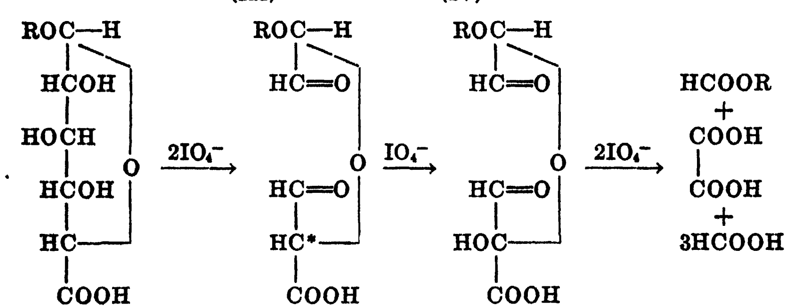
Benzimidazole	Maximum		Minimum	
	Wave-length	Log ε	Wave-length	Log ε
	A		*A*	
d-Gluco-	2800	3.78	2770	3.71
	2725	3.85	2590	3.61
d-Xylo-	2795	3.83	2765	3.79
	2725	3.88	2585	3.67
Anhydro-*d*-xylo-	2790	3.81	2765	3.74
	2730	3.86	2585	3.62

This structure was confirmed by periodate oxidation studies. 4 molar equivalents of periodate were consumed by II to produce 2-benzimidazole-carboxylic acid (V), formaldehyde, and 2 molar equivalents of formic acid. Assuming, as is concluded from the known periodate oxidations, that only the carbon to carbon bond of 1,2-glycols, 1,2-dicarbonyl compounds, and ketals is easily oxidized (4), the oxidation of II should stop with the production of the dialdehyde (III). If, however, the active hydrogen on the carbon atom between the aldehyde carbon and the imidazole ring were oxidized to a hydroxyl group, this intermediate (IV) would further be oxidized according to the orthodox pattern (V, etc.) with the consumption of a 3rd and 4th mole of periodate. To test the assumption that a configuration such as is present in III is oxidized by periodate, the behaviors of bornyl-*d*-glucuronide and methyl-*d*-galacturonide methyl ester were studied. If this assumption is true, the oxidation of bornyl-*d*-glucuronide should consume 5 moles of periodate (VII, etc.) instead of stopping after

the consumption of 2 moles and the production of VI. The carbon marked with an asterisk in VI is analogous to carbon atom 1′ in III in that it is situated between a carbon at the aldehyde stage of oxidation and a carbon at the carboxyl stage. 5 moles of oxidant were consumed by both the uronides. The glucuronide yielded bornyl formate and 5 molar equivalents of acid. Since 1 molar equivalent of oxalic acid was obtained, 3 molar equivalents of formic acid were produced. The oxidation products of the galacturonide were not investigated but 4 molar equivalents of acid were produced.



(III) (IV) (V)



(VI) (VII)

Evidence for this type of oxidation by lead tetraacetate in the sugar series has been reported by Hockett (5), who found that oxidation of ethyl-*β*-*d*-galactofuranoside does not stop at the hypothetical trialdehyde (VIII), but that another mole of oxidant is consumed presumably in replacing the active *α*-hydrogen by acetoxyl.

The kinetics of the oxidation of anhydro-*d*-xylo- and anhydro-*d*-arabobenzimidazoles by lead tetraacetate revealed that the arabo compound with adjacent *cis* hydroxyls was, as expected (6), oxidized faster during the first stages of the reaction than the xylo compound (see Fig. 1).

The 1′,4′-anhydro ring is also indicated by the conversion of these compounds by boiling acetic anhydride to 2-(α-furyl)benzimidazole (IX). This proof is valid only if no ring shift occurs during dehydration.

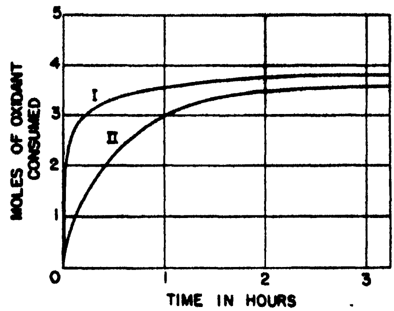
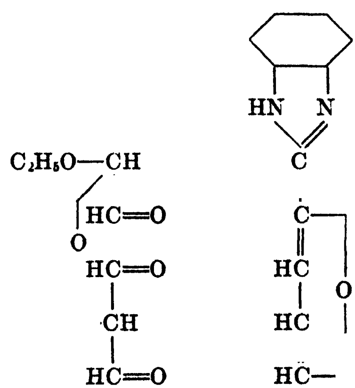


FIG. 1. Oxidation of anhydro-*d*-arabo-benzimidazole (Curve I) and anhydro-*d*-xylo-benzimidazole (Curve II) by lead tetraacetate in glacial acetic acid.

It should be noted that IX is not realized by refluxing compounds of the type I with acetic anhydride. Under milder acetylation procedures,



(VIII) (IX)

acetates of the aldo-benzimidazoles can be isolated. Thus *d*-arabo-benzimidazole yields a tetraacetate (7) and *d*-galacto-benzimidazole, a hexaacetate (8). *d*-Mannosaccharo-dibenzimidazole yields a hexaacetate and not a tetraacetate as first reported[4] (9).

It is of interest to note that under conditions of the O-acetyl determination of Wolfrom *et al.* (10), conditions which do not ordinarily hydrolyze N-acetyl groups, the N-acetyl of *d*-galacto-benzimidazole hexaacetate is

[4] *Analysis*—$C_{30}H_{30}O_{10}N_4$. Calculated, C 59.4, H 5.0, N 9.3; found, C 59.3, H 5.4, N 9.2.

completely hydrolyzed. The N-acetyls of *d*-mannosaccharo-dibenzimidazole hexaacetate are 70 per cent hydrolyzed. Blank determinations in acetone with phenolsulfonephthalein as an indicator showed that no acid is consumed by the unacetylated benzimidazoles.

EXPERIMENTAL

d-Xylo-benzimidazole Hydrochloride—The barium salt from the methanol-hypoiodite oxidation of 7.5 gm. (50 mM) of *d*-xylose was suspended in a small amount of water and decomposed with sulfuric acid. The barium sulfate was filtered and the solution concentrated to a syrup at reduced pressure. To the syrup dissolved in a mixture of 25 cc. of ethanol and 125 cc. of butanol, 4.6 gm. (25 mM) of *o*-phenylenediamine dihydrochloride[5] and 2.4 gm. (22 mM) of *o*-phenylenediamine (free base) were added and the mixture refluxed for 8 hours. From the cooled solution 11.7 gm. (85 per cent) of the hydrochloride of I crystallized, m.p. 176–178°. Recrystallization (with decolorization) from 12 parts of 95 per cent ethanol gave a mixture of anhydrous and apparently hydrated material from which the solvent was removed by drying at 110° *in vacuo*. The pure anhydrous hydrochloride was obtained by recrystallization from about 5 parts of absolute ethanol, m.p. 181–182°; $[\alpha]_D^{20} = +17.3°$ (C, 2; H_2O).

Analysis—$C_{11}H_{15}O_4N_2Cl$.	Calculated.	C 48.1, H 5.5, N 10.2
	Found.	" 47.8, " 5.6, " 10.2

d-Xylo-benzimidazole (I)—An aqueous solution of the hydrochloride was shaken with an excess of silver carbonate, filtered from the silver salts, treated with hydrogen sulfide, and again filtered. Concentration of the solution gave a syrup from which I crystallized. I was recrystallized from butanol, with the addition of acetone to give more complete crystallization. The melting point is 141–143°; $[\alpha]_D^{23} = +20.0°$ (C, 2; N HCl); $+19.7°$(C, 2; 5 per cent aqueous citric acid).

Analysis—$C_{11}H_{14}O_4N_2$.	Calculated.	C 55.4, H 5.9, N 11.8
	Found.	" 55.4, " 5.9, " 11.6

Anhydro-d-xylo-benzimidazole (II)—To 0.274 gm. (1 mM) of the hydrochloride of I were added 0.2 cc. (1 mM) of 5 M zinc chloride and 0.17 cc. (2 mM) of concentrated hydrochloric acid. The mixture was heated at 180° for 1½ hours. The amber-colored syrup was dissolved in about 3 cc. of water, filtered with the use of carbon (nuchar), and diluted to 5 cc. The

[5] 20 gm. of *o*-phenylenediamine were added to 150 cc. of absolute ethanol heated to boiling. 34 cc. of concentrated hydrochloric acid were added. The mixture was cooled in the refrigerator and the product filtered and washed with cold absolute alcohol and ether; 25.5 gm. were obtained.

specific rotation, usually about 62°, corresponds to a yield of about 96 per cent of II. Concentrated ammonium hydroxide (0.33 cc.) was added to the solution. Enough acetic acid was then added nearly to dissolve the precipitate which formed. The mixture was filtered hot after treatment with hydrogen sulfide. Concentrated ammonium hydroxide (0.33 cc.) was added and the solution was cooled in the refrigerator with occasional scratching, yield 0.19 gm. (86 per cent), m.p. 221–223°. Directions for further purification and preparation of secondary derivatives are given by Moore and Link (2). II may also be prepared directly from barium *d*-xylonate as described by them.

Analysis—$C_{11}H_{12}O_3N$.	Calculated.	C 60.0, H 5.4, N 12.7, mol. wt. 220
	Found.	" 60.0, " 5.6, " 12.8, 200–220 (Rast)

Anhydro-d-arabo-benzimidazole—This compound was obtained in comparable yields in a manner similar to that described for the preparation of II, m.p. 206–208°; $[\alpha]_D^{20} = +2.7$ (C, 2; 5 per cent aqueous citric acid).

Analysis—$C_{11}H_{12}O_3N_2$.	Calculated.	C 60.0, H 5.5, N 12.7
	Found.	" 59.8, " 5.5, " 12.7

Anhydro-d-lyxo-benzimidazole—*d*-Lyxo-benzimidazole (0.238 gm.) was converted to its anhydro compound by the procedure used to prepare II. A relatively small amount of the anhydro derivative crystallized out of the solution obtained upon filtration of the zinc sulfide, so that the mother liquors were concentrated to dryness. The residue was triturated with a few cc. of water, filtered, and washed to remove inorganic salts. The combined yield was 0.10 gm., m.p. 176–180°. After two recrystallizations from 3 cc. of ethanol, the melting point was 200–204°; $[\alpha]_D^{24} = +59.5°$ (C, 2; N HCl); +62.5° (C, 2; 5 per cent aqueous citric acid).

Analysis—$C_{11}H_{12}O_3N_2$. Calculated, N 12.7; found, N 12.8

The *picrate* melted at 132–138°.

Analysis—$C_{17}H_{15}O_{10}N_5$. Calculated, N 15.6; found, N 15.7

Anhydro-d-ribo-benzimidazole—*d*-Ribo-benzimidazole (11) (1.35 gm.) was converted to its anhydro product by the procedure given above. The aqueous solution obtained after the removal of the zinc sulfide was concentrated to dryness and the residue extracted with hot absolute ethanol. This extract was concentrated to dryness and the residue again extracted with 10 cc. of absolute ethanol. Absolute ether was added to the point of turbidity. The inorganic salts were removed and the filtrate concentrated to a volume of about 5 cc. After 3 days in the refrigerator, 0.3 gm.

of crystals had deposited. Two recrystallizations from ethanol gave a product melting at 82–83°; $[\alpha]_D^{25} = -84.5°$ (C, 2; ethanol). For analysis the compound was dried to constant weight at 65° over phosphoric anhydride *in vacuo*. The compound is extremely hygroscopic.

Analysis—$C_{11}H_{12}O_3N_2 \cdot 2H_2O$.	Calculated.	C 51.6, H 6.2, N 10.9
	Found.	" 51.4, " 5.4, " 10.6

When the sample was dried at 110° *in vacuo* for 24 hours, a glassy melt resulted. The loss in weight was 8.6 per cent, whereas theory calls for 14 per cent if the water is completely removed. The picrate of this transformation product melts at 120–125°.

Analysis—$C_{17}H_{15}O_{10}N_5$. Calculated, N 15.6; found, N 15.5

Permanganate Oxidation of I and II—The method used was that of Bistrzycki and Przeworski (12). To 5 mM of the aldo-benzimidazole and a few cc. of sodium carbonate solution in 100 cc. of hot water was added dropwise with stirring a solution of 7.9 gm. of potassium permanganate in 400 cc. of water, until a slight excess was present. This excess was removed by the addition of small quantities of the aldo-benzimidazole and the mixture was heated for ½ hour on the steam bath. The hot decolorized solution was acidified with acetic acid and allowed to cool, yielding crystalline 2-benzimidazolecarboxylic acid (V), m.p. 174° (decomposition). This compound was converted to benzimidazole by heating at 190° until the evolution of carbon dioxide had ceased. After recrystallization from water, the decarboxylation product melted at 171–172°, as did a mixture with benzimidazole prepared from formic acid and *o*-phenylenediamine. V was obtained from I and II in 70 per cent yields and from *d*-gluco-benzimidazole in 50 per cent yields.

Periodate Oxidation of d-Arabo-benzimidazole—Because of the insolubility of the aldo-benzimidazoles in water at room temperature, difficulty was experienced in carrying out oxidation studies. Therefore, a 35 per cent ethanol solution was used. Blank determinations showed that no detectable amounts of periodate were reduced by the solvent. *d*-Arabo-benzimidazole (0.522 gm., 2.195 mM) was dissolved in 50 cc. of hot water. 70 cc. of ethanol followed by 30 cc. of 0.427 M sodium periodate (5.82 molar equivalents) were added. The solution was diluted to 200 cc. with water. 20 cc. aliquots were removed for periodate determinations (13) after 24 and 36 hours. The consumption of oxidant was 3.1 and 3.3 molar equivalents (theory, 3). To a 40 cc. aliquot removed after 24 hours a few cc. of 10 per cent potassium nitrate were added to precipitate the excess periodate. Titration to a methyl red end-point showed the presence of 1.85 molar equivalents of acid (theory, 2).

A 10 cc. aliquot was removed after 24 hours for a formaldehyde determination. To this aliquot were added 3 cc. of phosphoric acid and enough 20 per cent sodium arsenite solution completely to reduce the iodate and periodate to iodide. This solution was then distilled under reduced pressure in the Kuhn-Roth micro acetyl apparatus, the solution being introduced continuously into the distillation flask. Near the end of the distillation more arsenite was added to reduce some iodine which formed. The receiver, cooled in ice, contained 25 cc. of water, to which had been added 2 cc. of an 8 per cent alcoholic solution of dimedon reagent and 5 cc. of N sodium acetate. After 24 hours in the refrigerator the formal-dimedon was filtered, weight 19.5 mg. (60 per cent for 1 molar equivalent of formaldehyde), m.p. 188–190°.

For the isolation of 2-benzimidazolealdehyde larger quantities of the aldo-benzimidazole were used. To a suspension of 2.4 gm. of *d*-arabo-benzimidazole in 50 cc. of water (for preparative purposes the more readily available *d*-gluco-benzimidazole can be used) a solution of 10.7 gm. of sodium periodate in 150 cc. of water was added. The suspension was shaken with cooling for 5 minutes and then kept at 4° overnight. The aldehyde was then filtered and the filtrate continuously extracted with ether for 1 week. More aldehyde separated out of the ether. Both crops were combined and washed copiously with water, weight 1.0 gm. (70 per cent), m.p. 235° (decomposition). Because of the great insolubility of this aldehyde in organic solvents no suitable recrystallization medium could be found.

Analysis—$C_8H_6ON_2$. Calculated, N 19.2; found, N 18.9

The *oxime* was prepared. After recrystallization from an ethanol-water mixture the melting point was 213–215°.

Analysis—$C_8H_7ON_3$. Calculated, N 26.1; found, N 25.8

The *dinitrophenylhydrazone* was prepared in an acetic acid medium. This derivative is extremely insoluble but could be recrystallized from a large volume of ethanol, m.p. 309–311°.

Analysis—$C_{14}H_{10}O_4N_6$. Calculated, N 25.8; found, N 25.9

Ultraviolet Absorption Spectra—The absorption spectrum of *d*-xylo-benzimidazole and of its transformation product was obtained from the aqueous solution and compared with that of *d*-gluco-benzimidazole. A solution of *d*-gluco-benzimidazole hydrochloride with the theoretical quantity of sodium bicarbonate to react with the hydrogen chloride was diluted to a concentration of 0.050 mM per liter. Similar solutions of *d*-xylo-ben-

zimidazole hydrochloride with sodium bicarbonate (concentration 0.054 mM per liter) and of anhydro-*d*-xylo-benzimidazole were prepared for the determination. The extinction coefficients showed two maxima and two minima, as indicated in Table I.

N-Benzyl-anhydro-d-xylo-benzimidazole — Anhydro-*d*-xylo-benzimidazole (0.90 gm.), 1 molar equivalent of a 1.5 N sodium methoxide solution, and 0.50 cc. of benzyl chloride were added to 25 cc. of absolute ethanol and refluxed for 12 hours. The mixture was concentrated to a few cc. and filtered. The crude benzyl derivative was washed with water and recrystallized from ethanol, m.p. 215–217°; yield 0.7 gm.

Analysis—$C_{18}H_{18}O_3N_2$. Calculated, N 9.0; found, N 9.0

Isopropylidene-anhydro-d-arabo-benzimidazole—To 1 gm. of the anhydro-arabo-benzimidazole suspended in 200 cc. of dry acetone was added 0.5 cc. of concentrated sulfuric acid. The mixture was shaken until solution was complete (2 days). Dry ammonia gas was used to neutralize the sulfuric acid. The ammonium sulfate was filtered, and the filtrate decolorized with carbon and concentrated to dryness *in vacuo*. The crystalline residue was recrystallized twice from water, yield 0.45 gm., m.p. 194–198°. This product contains 5 molecules of water of crystallization which may be removed at 100° *in vacuo* (calculated, H_2O 25.7 per cent; found, 26.0 per cent). The anhydrous product melts at 195–196°; $[\alpha]_D^{25} = -144°$ (*C*, 2; ethanol).

Analysis—$C_{14}H_{16}O_3N_2$. Calculated, N 10.8; found, N 10.8

Neutral Periodate Oxidation of Anhydro-d-xylo-, d-arabo-, and d-ribo-benzimidazoles—The anhydro-xylo derivative (0.490 gm.) was dissolved in 50 cc. of hot water and quickly cooled. Before crystallization occurred, 25 cc. of 0.427 M sodium periodate (4.8 molar equivalents) were added and the solution made up to 100 cc. After 24 hours, a 10 cc. aliquot showed a consumption of 3.99 molar equivalents of periodate (theory, 4). There was no change at 36 hours.

The determination of acid on a 10 cc. aliquot carried out as described above showed the presence of 2.4 molar equivalents of acid. Control titrations with methyl red as the indicator on a mixture of 2 moles of formic acid and 1 mole of 2-benzimidazolecarboxylic acid (V), the mixture which is theoretically present at the end of the oxidation, showed the presence of 2.7 moles of acid. Theory for the periodate oxidation is therefore 2.7 moles. V is 70 per cent neutralized at the methyl red end-point.

A formaldehyde determination on a 10 cc. aliquot conducted as described above yielded 34.4 mg. of the formal-dimedon derivative. This is 47 per cent of the amount calculated for 1 molar equivalent of formaldehyde.

The anhydro-*d*-arabo-benzimidazole (0.600 gm.) was treated with 30 cc. of 0.427 M sodium periodate (4.69 molar equivalents) in the manner described above. In 24 hours 4.10 molar equivalents of oxidant had been consumed and 2.4 molar equivalents of acid were produced. A 10 cc. aliquot gave 49.0 mg. of the formal-dimedon derivative. This is 46 per cent of the amount calculated for 1 molar equivalent of formaldehyde.

The *d*-ribo-benzimidazole transformation product (51.2 mg.) was treated with 11.5 molar equivalents of sodium periodate in 10 cc. of water. After 2 hours, 2-benzimidazolecarboxylic acid (V) began to crystallize out of the solution. In 4, 23, and 36 hours, 3.8, 4.0, and 5.0 molar equivalents of oxidant had been consumed. (This is based on a molecular formula $C_{11}H_{12}O_3N_2 \cdot 2H_2O$). After the periodate determinations were completed, the iodate was reduced with arsenite and the solution distilled into a receiver containing the dimedon reagent. A 35 per cent yield of the formal-dimedon was obtained.

Periodic Acid Oxidation of Anhydro-d-xylo-benzimidazole—The anhydrobenzimidazole derivative (1.1005 gm.) was dissolved in 71.9 cc. of 0.292 N periodic acid (4.2 molar equivalents) and made up to 100 cc. 3 minutes after solution the rotation was +0.75°. 7 hours later the value had fallen to +0.34° and crystals began to appear, making further observation of the rotation impossible. The solution was filtered at the 9th hour, the rotation being +0.27°, 3.33 molar equivalents of oxidant having been consumed. After 11, 24, 32, 48, and 56 hours the rotations were +0.23°, +0.17°, +0.10°, +0.08°, and +0.08°, and the molar equivalents of oxidant consumed were 3.43, 3.66, 3.74, 3.82, and 3.85, respectively.

The crystals of 2-benzimidazolecarboxylic acid (V) that separated were collected and washed with water, weight 0.601 gm. (79 per cent), m.p. 174° (decomposition). V was identified as described above.

Periodate Oxidation of Bornyl-d-glucuronide and α-Methyl-d-galacturonide Methyl Ester—To a solution of 0.6435 gm. of bornyl-*d*-glucuronide in 50 cc. of water were added 30 cc. of 0.433 M sodium periodate (7.21 molar equivalents) and 20 cc. of water. Periodate determinations at the expiration of $\frac{1}{2}$, 1, $4\frac{1}{2}$, 9, 14, and 38 hours showed the consumption of 2.96, 3.23, 4.38, 4.97, 4.97, and 5.04 molar equivalents of oxidant (theory, 5) and acid determinations showed the presence of 2.04, 2.42, 3.94, 4.85, 4.97, and 4.97 molar equivalents of acid (theory, 5).

For the isolation of oxalic acid, an oxidation was made as described above with 0.484 gm. of the glucuronide. The solution was extracted continuously with ether for 3 days. The ether was extracted with bicarbonate, and the extract acidified with acetic acid and treated with excess calcium chloride.

Calcium oxalate monohydrate (0.1655 gm., 85 per cent) was collected.

This amount of oxalate required 22.00 cc. of 0.0990 N permanganate, theory, 22.90 cc.

20 gm. of bornyl glucuronide in 2 liters of water at 4° were added to a solution of 60 gm. of sodium periodate in 2 liters of water, also at 4°, and kept at this temperature for 4 days. After a few hours droplets of bornyl formate began to appear. The mixture was extracted with ether, the ether washed with water, and the solution dried over sodium sulfate. The ether was removed and the residue distilled at 15 mm. The fraction distilling at 97–98° was collected, yield 4 gm.

Analysis—$C_{11}H_{18}O_2$. Calculated, C 72.5, H 9.9; found, C 72.3, H 10.1

α-Methyl galacturonide methyl ester hydrate (0.5085 gm.) was dissolved in 50 cc. of water and 30 cc. of 0.433 M sodium periodate (6.12 molar equivalents) and 20 cc. of water were added. At the expiration of ½, 1, 3, 7, 14, and 36 hours, 3.49, 3.80, 3.86, 4.39, 5.03, and 5.24 molar equivalents of periodate were consumed (theory, 5), with the production of 0.89, 1.05, 1.22, 2.27, 3.05, and 3.99 molar equivalents of acid (theory, 4).

Lead Tetraacetate Oxidation of 1′,4′-Anhydro-d-arabo- and xylo-benzimidazoles—The anhydro-benzimidazole derivative (0.1300 mg.) was weighed into a 200 cc. volumetric flask and dissolved in 10 cc. of glacial acetic acid. Then 190 cc. of 0.0662 M lead tetraacetate (21.3 molar equivalents) in glacial acetic acid were added with rapid mixing. Aliquots of 5 cc. were removed for the lead tetraacetate determinations made according to Hockett (6). In 3 minutes the arabo derivative consumed 2.5 molar equivalents of the oxidant and the xylo derivative 0.5. The arabo derivative had consumed 4.00 molar equivalents of the oxidant in 4 hours and the xylo derivative required 5 hours. In 96 hours both compounds had consumed 6 molar equivalents of oxidant and further consumption of oxidant was extremely slow. 6 molar equivalents of oxidant are required by theory if the 2 molar equivalents of formic acid are also oxidized. Both samples were oxidized simultaneously and kept at 20°.

Conversion of Anhydro-d-xylo-, arabo-, lyxo-, and ribo-benzimidazoles to 2-(α-Furyl)benzimidazole (IX)—The anhydro-benzimidazole derivative (0.50 gm.) was refluxed with 5 cc. of acetic anhydride for 2 hours. The solution was decolorized with carbon, concentrated *in vacuo* to 1 cc., and cooled in the refrigerator. Long needles of IX separated and were filtered and recrystallized from 50 per cent ethanol-water mixture, yield 0.20 gm., m.p. 286–290°. Comparable yields were obtained from all four of the anhydrobenzimidazoles. The melting point of a mixture of IX and the product prepared from furoic acid and o-phenylenediamine showed no depression. Weidenhagen (14) reports the melting point as 285–286°.

Analysis—$C_{11}H_8ON_2$. Calculated, N 15.2; found, N 15.2

IX is readily sublimed at about 150°. When *d*-arabo-benzimidazole was treated with acetic anhydride as described above, product IX was not obtained.

SUMMARY

1. Periodate and permanganate oxidation and ultraviolet absorption spectra of the product obtained by condensation of *d*-xylonic acid with *o*-phenylenediamine at 135° show it to be *d*-xylo-benzimidazole. Permanganate oxidation yielded 2-benzimidazolecarboxylic acid and periodate oxidation yielded 2-benzimidazolealdehyde, formaldehyde, and formic acid.

2. *d*-Xylo-, *d*-arabo-, *d*-lyxo-, and *d*-ribo-benzimidazoles are transformed by heating at 180° in the presence of zinc chloride and hydrochloric acid to yield 2-(1′,4′-anhydro-*d*-pento-tetrahydroxybutyl)benzimidazoles.

3. The structure of the anhydrides was indicated by their consumption of 4 moles of periodate to yield 2-benzimidazolecarboxylic acid, formaldehyde, and formic acid, and by their conversion to 2-(α-furyl)benzimidazole by heating with acetic anhydride.

4. The oxidations of the anhydro-aldopento-benzimidazoles by periodate have been interpreted to involve the oxidation of an α-hydrogen, activated by a carboxyl and an aldehyde group. This interpretation is substantiated by the behavior of bornyl-*d*-glucuronide which consumes 5 moles of periodate yielding bornyl formate, oxalic and formic acids.

BIBLIOGRAPHY

1. Moore, S., and Link, K. P., *J. Biol. Chem.*, **133**, 293 (1940).
2. Moore, S., and Kink, K. P., *J. Org. Chem.*, **5**, 637 (1940).
3. Elek, A., and Sobotka, H., *J. Am. Chem. Soc.*, **48**, 501 (1926).
4. Jackson, E. L., in Adams, R., Organic reactions, New York, **2**, 341 (1944).
5. Hockett, R. C., Nickerson, M. H., and Reeder, W. H., III, *J. Am. Chem. Soc.*, **66**, 472 (1944).
6. Hockett, R. C., Dienes, M. T., and Ramsden, H. E., *J. Am. Chem. Soc.*, **65**, 1474 (1943).
7. Ohle, H., *Ber. chem. Ges.*, **67**, 155 (1934).
8. Kuhn, R., and Bär, F., *Ber. chem. Ges.*, **67**, 898 (1934).
9. Lohmar, R., Dimler, R. J., Moore, S., and Link, K. P., *J. Biol. Chem.*, **143**, 551 (1942).
10. Wolfrom, M. L., Konigsberg, M., and Soltzberg, S., *J. Am. Chem. Soc.*, **58**, 490 (1936).
11. Dimler, R. J., and Link, K. P., *J. Biol. Chem.*, **150**, 345 (1943).
12. Bistrzycki, A., and Przeworski, G., *Ber. chem. Ges.*, **45**, 3483 (1912).
13. Fleury, P., and Lange, J., *J. pharm. et chim.*, **17**, 107 (1933).
14. Weidenhagen, R., *Ber. chem. Ges.*, **69**, 2263 (1936).

THE PRINCIPAL ALKALOIDS OF SABADILLA SEED AND THEIR TOXICITY TO MUSCA DOMESTICA L.*

By MIYOSHI IKAWA, ROBERT J. DICKE, THOMAS C. ALLEN, and KARL PAUL LINK

(*From the Departments of Biochemistry and Economic Entomology, College of Agriculture, University of Wisconsin, Madison*)

(Received for publication, March 19, 1945)

In 1819 Pelletier and Caventou announced the isolation of a basic substance from sabadilla seed (1) and later associated the name "veratrine" with this material (2). Veratrine, which is the term now used to describe the alkaloid mixture from sabadilla, has long been known for its medicinal uses and its toxicity to certain species of insects. The powdered seed itself has been tested and used as an insect repellant. In a recent review on the uses of sabadilla Allen *et al.* (3) reported the effectiveness of kerosene extracts of ground sabadilla seed (*Schoenocaulon* sp.) against the house-fly, *Musca domestica* L. The present work was undertaken to determine the constituents of the seed which are responsible for the toxicity.

Confusion exists in the early literature regarding the individual alkaloids present in sabadilla. A critical discussion of the early work is given by Wright and Luff (4). According to Henry ((5) pp. 628–632), cevadine, $C_{32}H_{49}O_9N$, and veratridine, $C_{36}H_{51}O_{11}N$, are present in the greatest quantity. In addition, the presence of cevadilline (sabadilline), $C_{34}H_{53}O_8N$ (4), and sabadine, $C_{29}H_{51}O_9N$ (6), has been reported. Of the alkaloids reported as present, cevadine and veratridine have been investigated most extensively. Although the general chemical nature of these alkaloids has been established, their exact structure is still unknown. An idea of their complexity can be obtained from the excellent fundamental work of Craig and Jacobs (7) on the structure of cevine, a common hydrolysis product of both cevadine and veratridine.

Though the sabadilla alkaloids are soluble in kerosene to only a limited extent (0.1 to 0.2 per cent), we have found that they are responsible for the toxicity of kerosene extracts of sabadilla seed to house-flies. The methods existing in the literature for the isolation of the individual alkaloids (4, 8) do not account for the bulk of the alkaloid mixture. Hence it appeared desirable to study the alkaloid mixture with other methods to realize a more complete recovery of the alkaloids and of the total toxicity.

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station, and supported through special grants from the Graduate Research Committee of the University, Office of Professor E. B. Hart, made available by the Wisconsin Alumni Research Foundation.

By passing a benzene solution of the alkaloid mixture through a column of activated aluminum oxide, then washing with benzene and eluting with chloroform and 95 per cent ethyl alcohol, it was possible to effect a partial separation into three fractions which differed in optical rotation and toxicity. By treating the benzene-soluble bases with dilituric acid (5-nitrobarbituric acid) according to Redemann and Niemann (9), the crystalline diliturate of cevadine was isolated together with some veratridine diliturate. From the chloroform eluate, which was the most toxic, veratridine diliturate was obtained by the same process. The free bases could readily be obtained from these derivatives by treatment with aqueous alkali and extraction with ether. The alcohol eluate was the least toxic fraction and no crystalline products could be isolated from it. The cevadine was identified by its hydrolysis to cevine and α-methylcrotonic acid (tiglic and angelic acid) after the procedure of Freund and Schwartz (10).

$$\underset{\text{Cevadine}}{C_{32}H_{49}O_9N} + H_2O = \underset{\text{Cevine}}{C_{27}H_{43}O_8N} + \underset{\alpha\text{-Methylcrotonic acid}}{C_5H_8O_2}$$

The veratridine was identified by its hydrolysis to cevine and veratric acid according to Blount (8):

$$\underset{\text{Veratridine}}{C_{36}H_{51}O_{11}N} + H_2O = \underset{\text{Cevine}}{C_{27}H_{43}O_8N} + \underset{\text{Veratric acid}}{C_9H_{10}O_4}$$

Veratridine proved to be very toxic, a solution of 42 mg. in 100 cc. of kerosene having an O. T. I. rating of +34.[1] Cevadine was fairly active, a solution of 80 mg. in 100 cc. of kerosene having an O. T. I. rating of +1. The bases (of the chloroform eluate) from the mother liquor after the isolation of veratridine diliturate were less active than the original alkaloid mixture, showing that the high toxicity of the chloroform eluate was due largely to veratridine. Cevine and cevine dibenzoate were both totally inactive in knockdown and kill. Veratridine at 20 mg. per 100 cc. and cevadine at 42 mg. per 100 cc. resulted in practically complete knockdowns in 3 minutes. Fig. 1 gives the concentration-mortality curves for veratridine, cevadine, and the alkaloid mixture. The curve for the pyrethrins is included for comparison. It shows that veratridine greatly surpasses the other substances in toxicity in kerosene to house-flies and that cevadine and the alkaloid mixture exhibit a slightly greater activity than the pyrethrins.

Sabadilla seed has a high oil content (18 to 19 per cent). When relatively high concentrations of the oil in kerosene were tested on the house-fly, high knockdowns but low mortalities were obtained. The knockdown

[1] The significance of the Official Test Insecticide rating is given in the section "Biological assay."

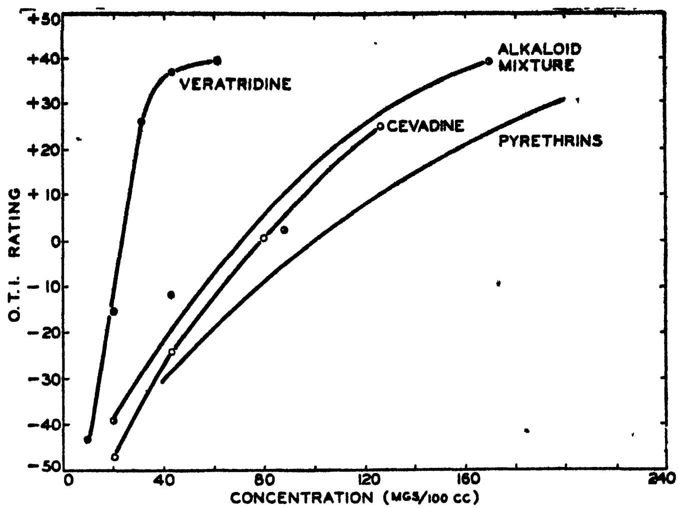


FIG. 1. Concentration-mortality curves of the sabadilla alkaloids in kerosene against the house-fly and a comparison with the pyrethrins.

TABLE I

Toxicity of Sabadilla Alkaloid Fractions and Sabadilla Oil in Kerosene to House-Fly

Substance tested	Kerosene	Toxicity: Per cent knockdown, 3 min.	Toxicity: Per cent knockdown, 10 min.	Toxicity: O.T.I. rating*
	mg. per 100 cc.			
Bases from soluble diliturates of $CHCl_3$ eluate	84	100	100	−21
	42	100	100	−23
Cevine	80 (Saturated solution)	0	0	−49
" dibenzoate	24–36 (Saturated solution)	0	0	−50
Sabadilla oil	2200	78	85	−47
	9600	88	97	−43
Oil extracted with HCl	50% solution (by volume)	0	0	−50
Alkaloids from sabadilla oil	84	100	100	−18
	170			+40
Kerosene		0	0	−50

* See the section on "Biological assay" for the method of testing and the significance of the Official Test Insecticide rating.

was eliminated when the oil was first extracted with dilute hydrochloric acid. From 100 gm. of sabadilla oil were isolated 1.2 gm. of alkaloids possessing a toxicity similar to that of cevadine (see Table I).

BIOLOGICAL ASSAY

The biological activity of the fractions was evaluated in kerosene as a contact insecticide to house-flies by a procedure previously described by Allen *et al.* (11). 3 cc. of a kerosene solution of the fraction to be tested were atomized into the upper end of a bell jar and flies raised under standard conditions were exposed to the settling mist for a period of 2 minutes. Both knockdown and kill were recorded.

To record relative toxicity, samples were compared with a standardized insecticide, Official Test Insecticide (O. T. I.). This insecticide is of such

TABLE II

Chromatographic Adsorption of Sabadilla Alkaloids on Aluminum Oxide

Fraction or eluate No.	Base	$[\alpha]_D^{20°}$ in alcohol	Toxicity in kerosene	
				O. T. I. rating*
	mg.		*mg. per 100 cc.*	
1 Benzene filtrate	824	+10.1	84	−16
			44	−38
2 " washing	389	+9.3	84	+20
			44	−29
3 " "	19			
4 $CHCl_3$ eluate	547	+8.1	86	+37
			44	−12
5 " "	329	+5.4	86	+25
			44	−6
6 " "	157			
7 " "	57			
8 " "	16			
9 Ethanol eluate	360	−6.7	86	−44
			44	−49
10 " "	58			
11 " "	18			

* See the section on "Biological assay" for the significance of the Official Test Insecticide rating.

strength that it is equivalent to a 50 per cent kill as described by Ford (12). The toxicity measurements in Tables I and II are given as O. T. I. ratings, which are calculated by subtracting the average per cent kill of the standard from that of the unknown. Thus a solution of low toxicity would have a negative rating, while one giving 40 per cent more kill than the standard insecticide is considered a +40 rating. The values given in Tables I and II represent the averages of six cages of thirty-five to forty flies per cage.

The kerosene used in the tests was a highly refined petroleum fraction known as "Sinclair insecticide oil base," obtained from the Sinclair Oil

Refining Company. As shown in Table I, the kerosene itself gives no kill and no knockdown.

The test samples were prepared by adding the sabadilla fractions to kerosene and heating on a steam bath to facilitate solution. The solutions were then cooled and filtered.

EXPERIMENTAL

Extraction of Sabadilla Seed with Petroleum Ether—The sabadilla seed was ground to 30 mesh and extracted with petroleum ether (Skellysolve A) in a Soxhlet extractor for 30 to 40 hours. The oil content was 18 to 19 per cent. It contains only traces of alkaloid.

Isolation of Alkaloid Mixture—The dried oil-free seed was extracted in a Soxhlet apparatus with acetone for 2 days. The acetone extract (from 1129 gm. of original seed) was concentrated to a syrup. The syrup was dissolved in 250 cc. of ethanol and the resulting dark solution poured into 4 liters of water containing 60 cc. of 10 per cent hydrochloric acid. The mixture was stirred well and filtered. Extraction of the filtrate with ether removed ether-soluble acidic and neutral substances. The aqueous phase was made alkaline with sodium hydroxide, whereupon a white precipitate of the alkaloids was obtained. The mixture was extracted with ether and the ether extract concentrated to dryness. By removing the last traces of ether *in vacuo*, the alkaloid mixture was obtained as a white fluffy amorphous mass. The average yield of alkaloid was 2.7 per cent of the original seed. The toxicity of the mixture is given in Table I and Fig. 1.

Fractionation of Alkaloid Mixture by Adsorption on Aluminum Oxide—3 gm. of the alkaloid mixture ($[\alpha]_D^{22°} = +2.9°$ in ethanol, $C = 8$) were dissolved in 100 cc. of benzene and passed through a column of activated aluminum oxide (22 $\times$ 240 mm.). The column was washed first with benzene (Fractions 1 to 3 (Table II)) until most of the benzene-soluble material had been removed from the column. The process was repeated with chloroform as the eluting agent (Eluates 4 to 8), followed by 95 per cent ethanol (Eluates 9 to 11). Successive 100 cc. fractions of the washings and eluates were collected, concentrated to dryness *in vacuo*, and the residues weighed. Table II lists the amounts obtained in each fraction, their specific rotations in ethanol, and their toxicities to house-flies. The total amount of material recovered from the column was 2.77 gm. (92.5 per cent).

Isolation of Cevadine—1 gm. of the combined benzene washings and 0.5 gm. of dilituric acid were dissolved in 40 cc. of boiling water. On cooling, a gum separated out which became crystalline after standing for 2 days. It was purified by recrystallization from water (Diliturate I). The yield was 0.9 gm. Diliturate I crystallizes with about 10 molecules of water in

pale yellow crystals which melt at 68–69°. On drying, it becomes bright yellow and the anhydrous product decomposes at 220° with previous darkening.

Analysis—Cevadine diliturate, $C_{32}H_{49}O_9N \cdot C_4H_3O_5N_3$
Calculated, C 56.53, H 6.85, N 7.33; found, C 56.33, H 6.72, N 7.29

Diliturate I (493 mg.) was added to 50 cc. of 1 per cent sodium hydroxide and the mixture shaken with ether. The solid gradually dissolved. The ether extract was concentrated to dryness and yielded 338 mg. of cevadine (89 per cent yield) ($[\alpha]_D^{21°} = +10.7°$ in ethanol, $C = 6.0$). Macbeth and Robinson (13) report $[\alpha]_D^{17°} = +12.5°$ in alcohol. The toxicity of the free base is given in Table I and Fig. 1.

The *aurichloride* crystallized from alcohol in fine yellow needles which melted with decomposition at 190°. Ahrens (14) reported a melting point of 182° with decomposition.

When the mother liquor from the crystallization of cevadine diliturate had been allowed to stand for 2 weeks, a white amorphous precipitate of veratridine diliturate was obtained which was recrystallized from water (yield, 0.2 gm.). Since larger amounts of veratridine diliturate were obtained from the chloroform eluate, it is described in the following section.

Proof of Identity of Cevadine—The base (520 mg.) from Diliturate I was hydrolyzed according to the directions of Freund and Schwartz (10). 226 mg. of crystalline *cevine* were obtained which sintered at 165° and were completely resinified at 172°. According to Freund and Schwartz (10), cevine sinters at 155–160° and becomes a transparent resin at 165–170°. The *dibenzoate* melted at 192–193°; Freund (15) reports 195–196°.

The mother liquor and washings from the isolation of the cevine were acidified with hydrochloric acid and extracted with ether. The ether extract was evaporated and the residue dissolved in 2 cc. of carbon disulfide containing a few drops of bromine. The mixture was allowed to stand for 2 days and the excess solvent and bromine were removed. After standing overnight, large crystals were obtained (36 mg.), melting at 86–87.5°. The dibromo derivative of *α-methylcrotonic acid* melts at 86–87° (15).

Isolation of Veratridine—1 gm. of the combined chloroform eluate and 0.5 gm. of dilituric acid were dissolved in 40 cc. of boiling water. On cooling, a gum separated which, after standing for 2 weeks, turned amorphous. The mother liquor was decanted and the residue recrystallized from water (Diliturate II). The yield was 0.4 gm. Diliturate II separates from water as a cream-colored powder which decomposes at 233° with previous darkening.

Analysis—Veratridine diliturate, $C_{36}H_{51}O_{11}N \cdot C_4H_3O_5N_3$
Calculated, C 56.73, H 6.43, N 6.62; found, C 56.30, H 6.36, N 6.67

Diliturate II (395 mg.) was added to 50 cc. of 1 per cent sodium hydroxide and the mixture shaken with ether. The solid gradually dissolved. The ether extract was concentrated to dryness and yielded 271 mg. of white amorphous veratridine (87 per cent yield) ($[\alpha]_D^{21°} = +7.2°$ in ethanol, $C = 3.9$). A sample reprecipitated from a solution of the hydrochloride had the indefinite melting point of 155–175°. Blount (8) reports the melting point of 160–180°, and an $[\alpha]_D^{22°}$ in alcohol of 8.0°. The toxicity of the veratridine is given in Table I and Fig. 1.

The mother liquor from the isolation of the veratridine diliturate was made alkaline with sodium hydroxide and extracted with ether. The toxicity of the alkaloid mixture so obtained is given in Table I.

Proof of Identity of the Veratridine—The base (212 mg.) from Diliturate II was hydrolyzed according to the direction of Blount (8). 91 mg. of crystalline *cevine* were obtained, which sintered at 162° and were completely resinified at 170°. The *dibenzoate* melted at 188–190°.

The base (144 mg.) was refluxed with 0.2 gm. of potassium hydroxide in 5 cc. of ethanol for 1 hour. The alkaline solution was concentrated to 2 cc., 25 cc. of water were added, and the mixture was acidified with hydrochloric acid and extracted with ether. The ether extract yielded *veratric acid*, which, when recrystallized from water, melted at 178–180° (29 mg.).

We are indebted to Dr. Walter A. Jacobs of The Rockefeller Institute for Medical Research, New York, for his generosity in supplying us with a sample of cevine, to Mr. N. J. Gothard, of the Sinclair Oil Refining Company, for confirming our results on the toxicity of cevadine and veratridine, and to our colleague, Mr. Frederick J. Dexheimer, for carrying out many of the biological assays.

SUMMARY

1. The alkaloids of sabadilla seed (*Schoenocaulon* sp.) are responsible for the toxicity of kerosene extracts of the ground seed to the house-fly, *Musca domestica* L.

2. By adsorption of the alkaloid mixture from sabadilla seed on a column of aluminum oxide, three fractions differing in toxicity were obtained. Cevadine and veratridine were isolated as their crystalline diliturates.

3. Veratridine in kerosene was found to be highly toxic to the house-fly. Cevadine exhibited less toxicity. Both alkaloids, as well as the remaining alkaloid mixtures, gave very quick and complete knockdowns.

4. Cevine and cevine dibenzoate showed no toxicity in kerosene to the house-fly. The oil extracted from sabadilla seed possessed knockdown but no killing power.

BIBLIOGRAPHY

1. Pelletier, P. J., and Caventou, J. B., *J. phys. chim., hist. nat. et arts*, **89**, 155 (1819).
2. Pelletier, P. J., and Caventou, J. B., *Ann. chim. et phys.*, **14**, 69 (1820).
3. Allen, T. C., Dicke, R. J., and Harris, H. H., *J. Econ. Entomol.*, **37**, 400 (1944).
4. Wright, C. R., and Luff, A. P., *J. Chem. Soc.*, **33**, 338 (1878).
5. Henry, T. A., The plant alkaloids, London, 3rd edition (1939).
6. *E. Merck's Jahresber.*, **4**, 3 (1890).
7. Craig, L. C., and Jacobs, W. A., *J. Biol. Chem.*, **141**, 253 (1941); **149**, 451 (1943).
8. Blount, B. K., *J. Chem. Soc.*, **122** (1935).
9. Redemann, C. E., and Niemann, C., *J. Am. Chem. Soc.*, **62**, 590 (1940).
10. Freund, M., and Schwartz, H. P., *Ber. chem. Ges.*, **32**, 800 (1899).
11. Allen, T. C., Dicke, R. J., and Brooks, J. W., *Soap and San. Chem.*, **19**, 94 (1943).
12. Ford, T. H., *Soap and San. Chem.*, **13**, 116 (1937).
13. Macbeth, A. K., and Robinson, R., *J. Chem. Soc.*, **121**, 1571 (1922).
14. Ahrens, F. B., *Ber. chem. Ges.*, **23**, 2700 (1890).
15. Freund, M., *Ber. chem. Ges.*, **37**, 1946 (1904).

INCREASED PLASMA FIBRINOGEN INDUCED BY METHYLXANTHINES*

BY JOHN B. FIELD, A. SVEINBJORNSSON, AND KARL PAUL LINK

(From the Department of Biochemistry, Wisconsin Agricultural Experiment Station, University of Wisconsin, Madison)

(Received for publication, March 19, 1945)

We have previously reported that the methylxanthines, caffeine (1,3,7-trimethylxanthine), theobromine (3,7-dimethylxanthine), and theophylline (1,3-dimethylxanthine), can induce a state of hyperprothrombinemia in the dog, rabbit, and rat (1). These substances also protect the same species against the hypoprothrombinemic action of the anticoagulant 3,3′-methylenebis(4-hydroxycoumarin) (1). The purpose of this report is to indicate that the administration of the methylxanthines in large single oral doses to the dog and rabbit raises the plasma fibrinogen level above the pre-test normal.

EXPERIMENTAL

The details of the animal experiments have already been described (1, 2). Fibrinogen was determined by a modification (3) of the colorimetric method of Folin and Ciocalteu (4), and total plasma protein by a standard colorimetric procedure (5). The findings from many trials in both the rabbit and dog will be reported in a highly condensed form.

Representative responses obtained by feeding single doses of caffeine, theophylline, and theobromine to dogs are given in Table I. It is noteworthy that, although the response obtained in dogs from a single dose was somewhat irregular, a consistent and more pronounced increase in fibrinogen levels was observed when the drugs are given on consecutive days. The following compounds, even when fed in relatively large doses, had no effect on the fibrinogen values in the dog: xanthine, adenine, guanine, uric acid, allantoin, creatine, creatinine, urea, guanidine, and uracil.

Representative results realized with rabbits are given in Table II. Caffeine, theophylline, theobromine, xanthine, adenine, uric acid, guanidine, and glycocyamine[1] induced detectable increases in the fibrinogen levels, while guanine, allantoin, urea, and 6-methyluracil had no effect.

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station, and supported through special grants from the Graduate Research Committee of the University, Office of Professor E. B. Hart, made available by the Wisconsin Alumni Research Foundation.

[1] This compound was kindly supplied by Professor Henry Borsook, California Institute of Technology, and we are indebted to Dr. R. T. Major, Research Department, Merck and Company, Inc., Rahway, New Jersey, for the 6-methyluracil.

In the course of this work the total plasma protein content was routinely estimated. Substantial daily variations were noted. But it should be indicated that the increased fibrinogen and prothrombin levels induced by

TABLE I

Representative Effects of Single Oral Dose of Methylxanthines on Fibrinogen Content of Dog Plasma

	Dose per kilo	Plasma fibrinogen levels				
		Normal and S.D.*	1 day after feeding	2 days after feeding	3 days after feeding	4 days after feeding
	mg.	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*
Caffeine	100	271 ± 18.0	405	340	288	
"	200	189 ± 8.0	186	394	332	276
Theophylline	200	156 ± 8.0	206	230	204	182
"	400	189 ± 13.0	308	232	285	
Theobromine	100	164 ± 7.0	191	237	245	218

* S.D. = standard deviation based on not less than four consecutive daily values obtained with the same dog prior to the test.

TABLE II

Representative Effects of Single Oral Dose of Methylxanthines on Fibrinogen Content of Rabbit Plasma

	Dose per kilo	Plasma fibrinogen levels					
		Normal and S.D.*	1 day after feeding	2 days after feeding	3 days after feeding	4 days after feeding	6 days after feeding
	mg.	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*
Caffeine	50	217 ± 5.0	398	487	742		
Theophylline	2.5	363 ± 6.0	535	491	539	458	451
"	50	270 ± 6.0	482	579	524	518	477
Theobromine	100	312 ± 13.0	524	572	606	637	637
Xanthine	50	322 ± 18.0	528	554	554	552	524
Adenine	50	340 ± 6.0	460	429	440	371	388
Uric acid	50	398 ± 7.0	551	470	461	429	399
Guanidine	50	365 ± 6.0	415	488	508	497	383
Glycocyamine	100	362 ± 7.0	398	508	429	431	374

* S.D. = standard deviation based on not less than four consecutive daily values obtained with the same rabbit prior to the test.

the methylxanthines are usually not reflected by significant increases in total plasma protein content. This is understandable, since fibrinogen and prothrombin constitute but a very small part of the total plasma protein.

DISCUSSION

The work of Tobitani (6) suggested that the methylxanthines have an effect on fibrinogen formation. He found that substances containing the guanidine nucleus shorten the coagulation time by not only increasing the formation of thrombin but also by simultaneously elevating the blood fibrinogen (6).

The present evidence on the influence of methylxanthines on the fibrinogen levels of the dog is supported by additional unpublished experiments.[2] In this study it was possible to maintain the pre-test fibrinogen levels within the normal range by the prophylactic administration of methylxanthines to dogs given toxic doses of chloroform. In the absence of methylxanthines, chloroform produces a marked diminution of plasma fibrinogen (7, 8).

The greater sensitivity of the rabbit to substances affecting the fibrinogen level merits comment. Detectable increases occurred in the rabbit after feeding as little as 1 to 2.5 mg. per kilo of the methylxanthines. The response of the dog was generally less consistent. A minimum dose of 75 to 100 mg. per kilo of the methylxanthine was required to produce a demonstrable effect. Xanthine, adenine, uric acid, guanidine, and glycocyamine significantly increased the plasma fibrinogen in the rabbit but not in the dog. Furthermore, in rabbits fed 25 to 50 mg. per kilo of the methylxanthines, the increase in fibrinogen values persisted for 2 to 3 weeks before pre-test levels were reestablished. This effect is detectable for only 3 to 4 days in the dog.

The possibility that the methylxanthines increase the fibrinogen level by causing hepatic injury was explored via the bromosulfalein retention test (9). No change from the normal retention was detected. It would therefore appear, as suggested previously ((1) p. 734), that the methylxanthines produce a stimulation of hepatic function which results in increased plasma fibrinogen.

SUMMARY

1. The oral administration of the methylxanthines, caffeine, theobromine, and theophylline, to dogs and rabbits raises the level of plasma fibrinogen. Xanthine, adenine, uric acid, guanidine, and glycocyamine increased the fibrinogen in the rabbit but not in the dog.

2. The rabbit was markedly more susceptible to the fibrinogen-increasing capacity of methylxanthines than the dog. Depending on the dose, an increased fibrinogen level may persist in the rabbit for 2 to 3 weeks.

[2] Field, J. B., Graf, L., and Link, K. P., in preparation.

BIBLIOGRAPHY

1. Field, J. B., Larsen, E. G., Spero, L., and Link, K. P., *J. Biol. Chem.*, **156**, 725 (1944).
2. Overman, R. S., Stahmann, M. A., Sullivan, W. R., Huebner, C. F., Campbell, H. A., and Link, K. P., *J. Biol. Chem.*, **142**, 941 (1942).
3. Reiner, M., Manual of clinical chemistry, New York (1941).
4. Folin, O., and Ciocalteu, V., *J. Biol. Chem.*, **73**, 627 (1927).
5. Greenberg, D. M., *J. Biol. Chem.*, **82**, 545 (1929).
6. Tobitani, T., *Okayama-Igakkai-Zasshi* (*Mitt. med. Ges. Okayama*), **51**, 816 (1939); *Chem. Abstr.* **37**, 681 (1943).
7. Whipple, G. H., and Hurwitz, S. H., *J. Exp. Med.*, **13**, 136 (1911).
8. Smith, H. P., Warner, E. D., and Brinkhous, K. M., *J. Exp. Med.*, **66**, 801 (1937).
9. Rosenthal, S. M., and White, E. C., *J. Am. Med. Assn.*, **84**, 1112 (1925).

PYRUVIC OXIDASE OF PROTEUS VULGARIS

By P. K. STUMPF*

(*From the Departments of Medicine and Biochemistry, College of Physicians and Surgeons, Columbia University, New York*)

(Received for publication, April 18, 1945)

Pyruvic acid enters into a wide variety of reactions in bacterial metabolism. In the presence of inorganic phosphate, cell-free extracts of *Lactobacillus delbrueckii* (1) oxidatively decarboxylate it to acetyl phosphate and CO_2, whereas an enzyme prepared from *Clostridium butylicum* (2) dissimilates it to acetic acid, H_2, and CO_2. *Escherichia coli* (3, 4) contains an enzyme which catalyzes the phosphorylative dissimilation of pyruvic acid to acetyl phosphate and formic acid; and *Aerobacter aerogenes* (5) contains an enzyme which induces the condensation of pyruvic acid to acetylmethylcarbinol with liberation of CO_2. In all four of these reactions inorganic phosphate is necessary. Lipmann (6, 7) has elucidated the nature of this phosphate requirement by showing that in the breakdown of pyruvic acid inorganic phosphate is taken up to yield acetyl phosphate.

The present investigation deals with a type of bacterial pyruvic oxidase which does not require inorganic phosphate and catalyzes the oxidation of pyruvic acid to acetic acid and CO_2 with liberation of much free energy (6, 8).

In carboxylases from yeast and animal sources, the prosthetic group is diphosphothiamine in conjunction with magnesium (9, 10). The pyruvic acid oxidase of *Lactobacillus delbrueckii* (1) contains flavin-adenine-dinucleotide as an essential component in addition to magnesium and diphosphothiamine, but this dinucleotide appears to be unnecessary for those pyruvic oxidases which do not require inorganic phosphate.

Proteus vulgaris contains an enzyme which, in the presence of diphosphothiamine and a bivalent metal ion, specifically catalyzes the oxidative decarboxylation of pyruvic acid to acetic acid and CO_2. Inorganic phosphate is not required in the reaction.

Preparation and General Properties—Fresh suspensions of *Proteus vulgaris* must be used for the preparation of an active enzyme. Bacterial suspensions, stored at about $-50°$ for a period of several months, yield inactive preparations.

Two methods for disintegrating bacteria were employed; namely, expo-

* From a dissertation submitted by the author in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University. This work has been supported by a grant from the John and Mary R. Markle Foundation administered by Dr. D. E. Green.

sure to ultrasonic irradiation (11) and crushing by the Booth-Green mill (12). After either of the two treatments, intact cells and cellular débris were removed by repeated centrifugation. In both cases the supernatant juice was opalescent and pale yellow in color. The enzyme could be concentrated with little loss in activity by precipitation with acetic acid at pH 4.3. A concentrated enzyme solution showed large losses of activity when held at about −50° for as little as 24 hours, but at 4° was stable over a period of several weeks.

The enzyme tolerates exposure to 60° for 5 minutes at pH 7 with little loss in activity, but exposure to 70° and 75° for the same period leads respectively to 50 per cent and 100 per cent inactivation. Dialysis against distilled water or 0.1 per cent saline causes no loss in activity, whereas dialysis against 0.02 M phthalate buffer at pH 4.0 or against 0.02 M phosphate or 0.02 M pyrophosphate buffer at pH 8.8 is accompanied by complete

TABLE I

Fractional Centrifugation of Enzyme

Stage	Gravitational field	Centrifugation time	$Q_{O_2}^{N}$*
	g	*min.*	
1. Fresh cell suspension			325
2. Supernatant after ultrasonic disintegration and 1st centrifugation	2,500	30	102
3. Supernatant of (2) after 2nd centrifugation	9,700	60	288
4. Sediment of (2) after 2nd centrifugation	9,700	60	50
5. Supernatant of (3) after 3rd centrifugation	100,000	45	0
6. Sediment of (3) after 3rd centrifugation	100,000	45	410

* C.mm. of oxygen per hour per mg. of bacterial nitrogen.

loss of activity which is not restored by the addition of boiled bacteria, boiled enzyme, yeast extract, or flavin-adenine-dinucleotide. Acetone precipitation in the cold (−10° at pH 7) inactivated the enzyme completely. Exposure of the enzyme preparation to ultrasonic irradiation for 30 minutes causes no inactivation.

The enzyme appears to be associated with macro particles which are sedimented in strong centrifugal fields (see Table I). It has an optimum pH ranging from 5.5 to 6.1, depending on the buffer employed (Fig. 1). Activity in the three buffers tested, acetate, phosphate, and phthalate, falls sharply below pH 5.3 and above 6.7. In phosphate buffer the enzyme is completely inactive above pH 7.6. Below pH 5.3 there is considerable aggregation of particles.

Enzyme activity was followed for the most part manometrically. Substitution of oxygen for air in the gas phase had no influence on the rate of

oxygen consumption, indicating that the limiting reaction was not the rate of oxidation of the reduced enzyme by oxygen. In the presence of pyruvic acid, the enzyme system slowly reduced methylene blue anaerobi-

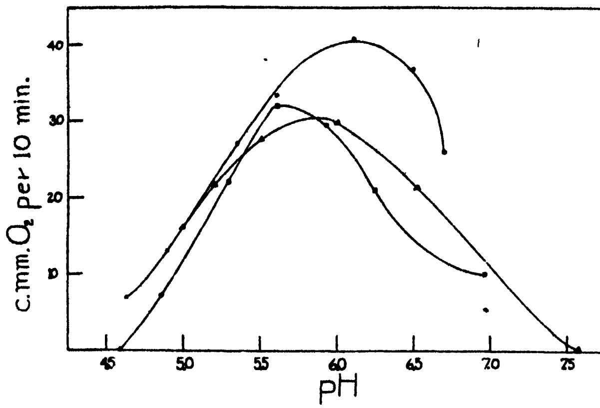


FIG. 1. Reaction velocity as a function of pH. Each manometer vessel contained 0.5 cc. of enzyme, 0.5 cc. of 0.2 M lithium pyruvate, 0.5 cc. of 0.5 M buffer, 0.1 cc. of 0.2 per cent manganese sulfate, 0.1 cc. of 0.1 per cent diphosphothiamine. Final volume 3 cc.; NaOH in center pot; temperature 38°. The following symbols indicate the nature of the buffer; ● acetate, ▲ phosphate, ■ phthalate.

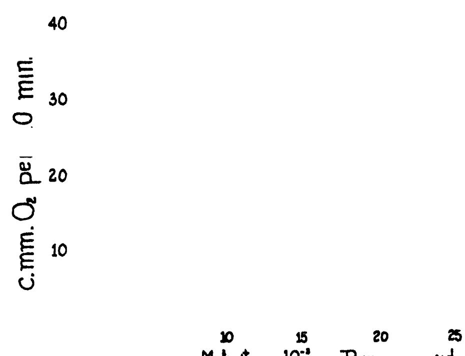


FIG. 2. Reaction velocity as a function of substrate concentration. Each manometer vessel contained 0.5 cc. of enzyme, 0.1 cc. of 0.2 per cent manganese sulfate, 0.1 cc. of 0.1 per cent diphosphothiamine, 0.5 cc. of 0.5 M acetate buffer at pH 6.0. Final volume 3 cc.; NaOH in center pot; temperature 38°.

cally. As an electron acceptor, however, methylene blue is only 11 per cent as efficient as oxygen.

Fig. 2 shows the relation between reaction velocity and substrate concentration. The molar concentration of substrate at which half the maximum

velocity is reached is about 5×10^{-3}. Above 20×10^{-3} M, increase in the concentration of substrate does not lead to any change in the velocity of oxidation.

Reaction—The oxidative decarboxylation of pyruvic acid follows the equation

$$(1) \qquad CH_3COCOOH + \tfrac{1}{2}O_2 \rightarrow CH_3COOH + CO_2$$

Anaerobically, no disappearance of pyruvic acid has been observed. Either anaerobically or aerobically, the addition of methylene blue, riboflavin, flavin-adenine-dinucleotide, or inorganic phosphate did not alter the nature of the reaction. It therefore seems improbable that either a hydroclastic reaction[1] or a dismutation takes place as an intermediary step. Since a possible condensation product, acetylmethylcarbinol, and its oxidation and reduction products, diacetyl and 2,3-butylene glycol, were found to be completely unreactive in the presence of the enzyme system, it would also seem improbable that a preliminary condensation of 2 molecules of pyruvic acid with subsequent oxidative disruption of the labile condensation product to 2 molecules of acetic acid and CO_2 could take place. The possibility that the actual mechanism might be a simple two-step reaction, the first step being a decarboxylation and the second a rapid oxidation of the formed aldehyde, is excluded because CO_2 is not produced under anaerobic conditions and the enzyme does not oxidize acetaldehyde. Finally, the hypothesis of a condensation of pyruvic acid with specific amino groups to form a Schiff base, which is then rapidly disrupted to acetic acid, CO_2, and the regenerated amino group, has been considered, but available experimental data do not justify a decision concerning its validity.

The evidence at hand merely suggests that, since phosphate is not involved in the reaction, the pyruvic acid possibly forms an unstable hydrate which is dehydrogenated to acetic acid and CO_2 in the presence of the specific enzyme system and oxygen.

There is no evidence as to the type of electron acceptor immediately responsible for the oxidation of the substrate. Since magnesium and zinc are effective bivalent cations, the rôle of manganese as a possible electron acceptor must be ruled out. The possibility that the cytochrome system acts as a bridge between the substrate and oxygen is likewise excluded, since sodium azide (final concentration 1 M) (13) does not inhibit oxygen uptake. Finally, application of the methods of separating reversibly the prosthetic group from the protein moiety of the enzyme, as developed by

[1] Utter and Werkman (4) limit the hydroclastic reaction to the anaerobic enzymatic disruption of pyruvic acid to formic and acetic acids by either water or phosphoric acid.

Warburg and others (14, 15), has yielded either a partially or completely inactive enzyme, the activity of which could not be restored on addition of flavin-adenine-dinucleotide, yeast extract, boiled bacteria, or boiled enzyme.

TABLE II

Components of System

The complete system contained 1.5 cc. of enzyme, 0.5 cc. of 0.5 M phosphate buffer at pH 6.0, 0.2 cc. of 0.1 per cent diphosphothiamine, 0.1 cc. of 0.2 per cent manganese sulfate, 0.5 cc. of 0.2 M lithium pyruvate, and water to make a final volume of 3.0 cc.; NaOH in center pot; temperature 38°.

	Oxygen uptake in 30 min.
	c.mm.
Complete system	157
Without diphosphothiamine	0
" manganese	30
" substrate	0
" phosphate	152
With flavin-adenine-dinucleotide	156
" boiled enzyme	0

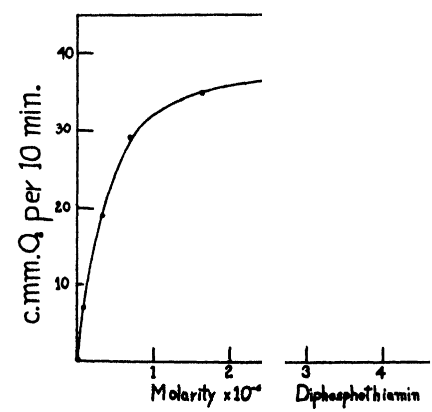


FIG. 3. Reaction velocity as a function of diphosphothiamine concentration. Each manometer cup contained 0.5 cc. of enzyme, 0.5 cc. of 0.5 M acetate buffer at pH 6.0, 0.1 cc. of 0.2 per cent manganese sulfate, 0.5 cc. of 0.2 M lithium pyruvate. Final volume 3 cc.; NaOH in center pot; temperature 38°.

Components—The pyruvic acid oxidase system consists of at least (1) a specific protein, (2) diphosphothiamine, and (3) a bivalent cation such as manganese (Table II).

Diphosphothiamine is the specific coenzyme. Fig. 3 shows the relationship between the concentration of diphosphothiamine and the reaction

velocity. Thiamine, monophosphothiamine, and pyrithiamine neither replace diphosphothiamine nor inhibit the reaction in which diphosphothiamine participates. Flavin-adenine-dinucleotide, adenine pyrophosphate, and coenzyme 1 also cannot replace the prosthetic group.

Evidently the pyrophosphate group of diphosphothiamine plays an important rôle in linking the prosthetic group to the enzyme protein. Inorganic pyrophosphate inhibits the activity of the enzyme when present in adequate amounts, but this inhibition can be reversed by increasing the concentration of diphosphothiamine (Table III). Thus, a concentration of pyrophosphate 250 times that of diphosphothiamine brings about 91

TABLE III

Effect of Pyrophosphate on Enzyme Activity

Each manometric cup contained 0.5 cc. of enzyme (1.46 mg. of bacterial N), 0.5 cc. of 0.2 M lithium pyruvate, 0.05 cc. of 0.2 per cent manganese sulfate, 0.5 cc. of 0.5 M acetate buffer at pH 6.0, 0.2 M pyrophosphate at pH 6.0 of varying amounts, 0.1 per cent of diphosphothiamine of varying amounts (at pH 6.0), and water to make a total volume of 3.0 cc.; NaOH in center pot; temperature 37°.

Pyrophosphate	Diphosphothiamine	Oxygen uptake	Per cent inhibition
$M \times 10^{-3}$	$M \times 10^{-6}$	*c mm. per 10 min.*	
	34.5	43	
0.7	34.5	18	58
3.4	34.5	6	86
6.7	34.5	3	92
13.4	34.5	2	96
3.4	14.0	4	91
3.4	34.0	5	88
3.4	69.0	9	79
3.4	207	18	58
3.4	415	29	32
3.4	690	32	26

per cent inhibition. If the concentration of diphosphothiamine is raised to a level one-fifth that of pyrophosphate, inhibition becomes negligible. In adequate amounts manganese, which forms an insoluble pyrophosphate, also abolishes pyrophosphate inhibition.

Diphosphothiamino enzymes can be classified into two general groups, depending on the relative degrees of dissociation of the conjugated proteins. In the first group of diphosphothiamino enzymes of which yeast carboxylase (9), pyruvic oxidase of *Lactobacillus delbrueckii* (1), the phosphoroclastic enzyme of *Escherichia coli* (3), pyruvic ketolase, and α-ketoglutaric carboxylase of animal tissues (10) are examples, the coenzyme is firmly combined with the protein in neutral solution. Dissociation, however, takes place

readily below pH 4.6 or above pH 8. In the second group, of which acetaldehyde ketolase is an example (10), the coenzyme is completely dissociated even in neutral solutions. Since the supernatant juice obtained by centrifuging suspensions of disintegrated *Proteus vulgaris* has been found consistently not to catalyze the oxidation of pyruvic acid unless supplemented with diphosphothiamine, the inference might be drawn that this pyruvic oxidase is a highly dissociated diphosphothiamino protein, and that in the process of preparing the cell-free enzyme the dissociated coenzyme is diluted sufficiently to mask its action. If such were the case, then disintegrating a thick paste of bacteria (500 mg. dry weight per cc.) rather than the usual

TABLE IV

Inactivation of Diphosphothiamine by Proteus vulgaris

Dephosphorylation of diphosphothiamine was demonstrated by incubating 100 γ of diphosphothiamine with 1 cc. of fresh *Proteus vulgaris* suspension in 0.5 cc. of 0.5 M acetate buffer at pH 6.0 for 4 hours at 37°. Reaction mixtures were made up to a volume of 10 cc., bacteria were removed by centrifugation, and aliquots determined for thiamine and diphosphothiamine by the thiochrome method.

	System	Diphosphothiamine	Thiamine	O_2 uptake with pyruvic oxidase
		γ	γ	*c.mm. per 10 min.*
1.	Diphosphothiamine + *P. vulgaris*	0	97	0
1a.	" + polidase*	0	98	
2.	" + boiled *P. vulgaris*	98	0	28
2a.	" + " " + polidase	0	99	
3	Cell-free pyruvic oxidase preparation + diphosphothiamine	99	0	30
4.	Thiamine + *P. vulgaris*	0	98	
5.	" + boiled *P. vulgaris*	0	99	

* *Cf.* foot-note 6.

thin suspension (25 mg. dry weight per cc.) would be expected to minimize the dilution of coenzyme. Nonetheless, the supernatant juice, obtained by centrifuging down disintegrated bacteria, is still inactive unless diphosphothiamine is added. Moreover, whereas addition of the coenzyme to the bacterial suspension prior to disintegration should nullify the dilution effect, the requirement of the enzyme for diphosphothiamine is the same whether or not diphosphothiamine is added to bacterial suspensions prior to disintegration. On the whole the evidence favors the view that, in the process of preparing the cell-free enzyme, destruction of diphosphothiamine takes place.

As summarized in Table IV, the intact bacterial cell, but not cell-free

preparations, contains a phosphatase which rapidly dephosphorylates diphosphothiamine to thiamine.[2] Thiamine itself is not further attacked. This bacterial phosphatase probably only attacks diphosphothiamine when it is free and not when bound to a protein, since suspensions of *Proteus vulgaris* do not inactivate yeast carboxylase. It is therefore permissible to conclude that some dissociation of the oxidase must take place, but the precise degree of dissociation has yet to be determined. The sequence of events in the inactivation of the supernatant juice is then probably (*a*) dephosphorylation of dissociated diphosphothiamine[3] and (*b*) further dissociation of the enzyme to satisfy the equilibrium requirements. Eventually all the coenzyme would be inactivated in this manner. If measures could be taken to eliminate phosphatase action, it would be possible to estimate the degree of dissociation of the coenzyme. The following procedures were explored as means of preventing phosphatase action, but in no case with any success: (1) disintegration in the presence of a high concentration of fluoride (0.03 M), (2) rapid disintegration at 2° and prompt removal of bacteria by high speed centrifugation at 4° (the over-all time to prepare the cell-free extract being not more than 25 minutes), and (3) disintegration in acid solution (pH 5), in alkaline solution (pH 8), or in ammonium sulfate solution (20 per cent).

In addition to diphosphothiamine, a bivalent metal is essential for the activity of the enzyme. The bivalent metal is firmly bound to the protein but can be completely split off by exposure to acid (pH 4.0) for about ½ hour at 0°. Other methods such as repeated precipitation by acetic acid, prolonged dialysis against distilled water, or acid or alkaline buffers fail to in-

[2] Phosphatase action by whole *Proteus vulgaris* was demonstrated by incubating diphosphothiamine with fresh bacteria under defined conditions (Table IV). The bacteria were spun down and aliquots of the reaction mixtures were estimated for thiamine and diphosphothiamine by the thiochrome method. Boiled controls showed no spontaneous breakdown of diphosphothiamine. The presence of diphosphothiamine was also confirmed by an equally sensitive test; *viz.*, the addition of aliquots of the reaction mixtures to the pyruvic acid oxidase system complete but for diphosphothiamine. Oxidation of pyruvic acid, as indicated by an oxygen uptake, revealed the presence of at least 3 γ of diphosphothiamine per cc. of reaction mixture.

[3] Since oxidation of pyruvic acid by the intact bacterial cell is not enhanced by the addition of diphosphothiamine, it is conceivable either that free diphosphothiamine is present in excess in the bacterial cell or that the oxidase is not dissociated appreciably within the cell. There is furthermore the dilemma that, whereas diphosphothiamine added to a suspension of bacteria is rapidly dephosphorylated, the diphosphothiamine present in association with the oxidase appears to be stable as long as the cell is intact. Probably pyruvic oxidase and the phosphatase do not come into contact with one another within the cell, although diphosphothiamine added to the external medium freely enters the cell and comes into rapid contact with the phosphatase.

duce complete dissociation of the enzyme or bring about partial or complete inactivation. Calcium, barium, cadmium, aluminum, and ferric ions are

TABLE V

Effect of Metal Cations on Enzyme Activity

Each manometer cup contained 0.5 cc. of enzyme (prepared as described in the text), 0.5 cc. of 0.1 per cent diphosphothiamine, 0.5 cc. of 0.5 M acetate buffer at pH 6.0, 0.5 cc. of 0.2 M lithium pyruvate, 0.1 cc. of 0.015 M metal cation, and water to make a total volume of 3.0 cc.; NaOH in center pot; temperature 37°.

Metal cation	Final concentration	Relative activity
	moles per l. $\times 10^{-4}$	
Manganese	0.45	100
	4.5	100
Magnesium	5.0	79
Calcium	5.0	0
Barium	5.0	0
Iron (ous)	5.0	67
Iron (ic)	5.0	0
Cobalt (ous)	5.0	45
Nickel (ous)	5.0	67
Cadmium	5.0	0
Zinc	5.0	60
Aluminum	5.0	0

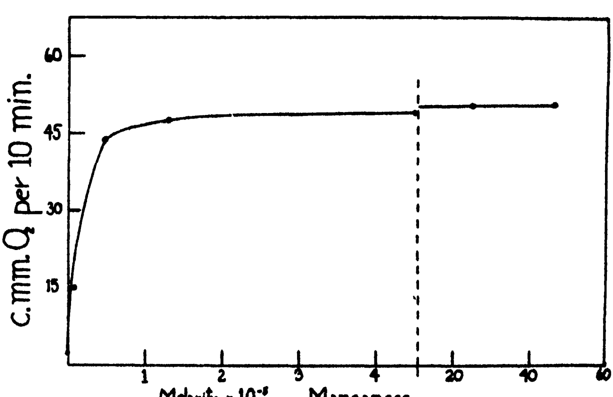


FIG. 4. Reaction velocity as a function of the concentration of manganese sulfate. Each manometer cup contained 0.5 cc. of enzyme, 0.5 cc. of 0.2 M lithium pyruvate, 0.1 cc. of 0.1 per cent diphosphothiamine, 0.5 cc. of 0.5 M acetate buffer at pH 6.0. Total volume 3 cc.; NaOH in center pot; temperature 38°.

ineffective, but magnesium, ferrous, nickel, zinc, and cobalt ions can replace manganese qualitatively (Table V). The relationship between the reaction velocity and the concentration of manganese is shown in Fig. 4.

The natural bivalent metal of the enzyme system probably is magnesium, since traces of the metal can be detected by the quinalizarin test (16) in ashed enzyme preparations. Moreover, magnesium is found far more abundantly in bacteria than are other bivalent metals (17).

The fact that three components, *viz.* protein, a bivalent metal, and diphosphothiamine, are required provides some basis for visualizing the structure of the system. Magnesium or other suitable bivalent metal ions can be considered as a bridge connecting thiamine pyrophosphate to the specific protein in the manner suggested in the formulation, protein-magnesium-thiamine pyrophosphate. The hypothetical bridge cannot be

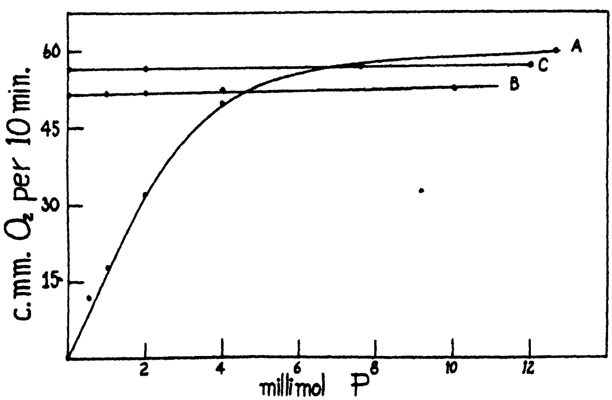


FIG. 5. Reaction velocity as a function of phosphate concentration. Each manometer cup contained 0.5 cc. of enzyme, 0.5 cc. of acetate buffer, increasing amounts of 0.5 M phosphate buffer at pH 6.0, 0.1 cc. of 0.2 per cent manganese sulfate, 0.1 cc. of 0.1 per cent diphosphothiamine, 0.5 cc. of 0.2 M lithium pyruvate. Final volume 3 cc.; NaOH in center pot; temperature 38°. Curve A represents pyruvic oxidase of *Lactobacillus delbrueckii*, Curve B that of *Escherichia coli*, and Curve C that of *Proteus vulgaris*.

thiamine pyrophosphate, since preparations can be obtained which contain the metal but which are completely free of the coenzyme. Moreover, inhibition by inorganic pyrophosphate can be readily ascribed to its reversible union with the magnesium-protein complex, thus blocking a similar union with thiamine pyrophosphate. The importance of the pyrophosphate group of thiamine pyrophosphate is further emphasized by the inactivity of the monophosphoric ester of thiamine as the prosthetic group.

The enzymatic oxidation of pyruvic acid proceeds as well in the absence as in the presence of inorganic phosphate in concentrations of the same order as that required for maximum activity of the pyruvic acid oxidase of *Lactobacillus delbrueckii* (1) (*cf.* Fig. 5). Similarly, the pyruvic acid oxidase of

Escherichia coli (18) does not require the presence of phosphate. In his studies on the oxidase of this bacterium, Still (18) noticed that the addition of phosphate buffer of pH 6.0 greatly increased the rate of oxidation of pyruvic acid. This apparent phosphate effect can, however, be attributed to the decrease in pH which resulted on addition of this acid buffer to his unbuffered enzyme solutions. Presumably his enzyme solution had, prior to the addition of buffer, a pH value of about 7, at which level the activity of the enzyme is only small compared to that at pH 6.0. In the present experiments, when the reaction mixtures were maintained at pH 6.0 with 0.5 M acetate buffer, activity was practically the same with or without phosphate (Fig. 5).

It might be assumed that trace amounts of inorganic phosphate react with pyruvic acid to form acetyl phosphate, which in turn is immediately

TABLE VI

Acetyl Phosphate Formation

Each manometer cup contained 0.5 cc. of enzyme, 0.1 cc. of 0.2 per cent manganese sulfate, 0.5 cc. of 0.5 M acetate buffer at pH 6.0, 0.3 cc. of M phosphate at pH 6.0, 0.5 cc. of M lithium pyruvate, 0.1 cc. of 0.1 per cent diphosphothiamine, and water to a total volume of 3.0 cc.; NaOH in center pot; temperature 37°. Acetyl phosphate estimated by the hydroxamic acid method of Lipmann.

Enzyme source	Oxygen uptake	Pyruvate disappearance	Acetyl phosphate formed
	micromoles	*micromoles*	*micromoles*
I. *Proteus vulgaris*	2.0	4.1	0
II. *Escherichia coli*	6.2	12.1	0
III. *Lactobacillus delbrueckii*	6.1	12.3	6.0
I + III	8.3	16.1	3.2
II + "	11.9	23.7	3.8

broken down to acetic acid with a regeneration of inorganic phosphate, the whole process being catalyzed by an extremely active acetyl phosphatase. In the light of Lipmann's findings (7), that 0.03 M fluoride inhibits the breakdown of acetyl phosphate by at least 50 per cent, one would expect that in the presence of fluoride any traces of phosphate would soon accumulate as organically bound phosphate, and the reaction would come to a standstill. However, 0.03 M fluoride does not inhibit the reaction either initially or after the reaction has proceeded for several hours. Utter and Werkman (4) and Lipmann[4] have recently demonstrated that the decomposition of acetyl phosphate is largely prevented by a high concentration of phosphate (0.1 M), with consequent accumulation of the otherwise labile product. As summarized in Table VI, in the presence of a final phosphate concentra-

[4] Lipmann, F., private communication.

tion of 0.1 M, no acetyl phosphate accumulated in the oxidative breakdown of pyruvic acid catalyzed by the oxidase of either *Proteus vulgaris* or *Escherichia coli*,[5] whereas with the oxidase of *Lactobacillus delbrueckii* as catalyst, 98 per cent of the calculated amount of acetyl phosphate formed was found at the conclusion of the reaction. If the enzyme preparations of both *P. vulgaris* and *E. coli* contain extremely active acetyl phosphatases, which act regardless of the phosphate concentration, they should, in the presence of the enzyme preparations of *L. delbrueckii*, prevent the accumulation of acetyl phosphate. Experiment has shown that in the presence of either *P. vulgaris* or *E. coli* a considerable proportion of the acetyl phosphate formed by the *L. delbrueckii* enzyme during the oxidation of the keto acid was not decomposed. While an acetyl phosphatase is undoubtedly present in both

TABLE VII

Specificity

Each manometer cup contained 0.25 cc. of enzyme, 0.5 cc. of 0.2 M substrate, 0.1 cc. of 0.2 per cent manganese sulfate, 0.5 cc. of 0.5 M acetate buffer at pH 6.0, water to make a total volume of 3.0 cc.; NaOH in center pot; temperature 37°.

Substrate	Without diphosphothiamine	With diphosphothiamine
	c.mm. per 10 min.	*c.mm. per 10 min.*
Formic acid	24	23
Glyoxylic acid	0	0
Pyruvic acid	0	22
α-Ketobutyric acid	0	0
α-Ketocaproic "	0	0
α-Ketoglutaric acid	0	0
Phenylpyruvic acid	4	5
Lactic acid	0	0
Acetoacetic acid	0	0

P. vulgaris and *E. coli* enzyme preparations, its action is too slow to account for the absence of acetyl phosphate as a reaction product of pyruvate oxidation by these two oxidases.

Specificity—The enzyme oxidizes only pyruvic acid. Since at pH 5.8 the keto and enol forms of pyruvic acid (19) are present in equal amounts, it is uncertain which of the two is the reactive form.

Table VII contains a summary of the compounds which have been tested. α-Ketobutyric, α-ketocaproic, α-ketoglutaric, acetoacetic, glyoxylic, and lactic acids are not oxidized. Since the enzyme preparation oxidizes formic

[5] *Escherichia coli*, grown on an agar surface in Roux bottles, does not contain the phosphoroclastic enzyme of Werkman *et al.* (3), and hence the possibility of acetyl phosphate formation by this enzyme is eliminated.

and phenylpyruvic acids under conditions (in the absence of diphosphothiamine) in which pyruvic acid is not oxidized, the oxidation of these two acids cannot be attributed to pyruvic acid oxidase.

Inhibitors—With 0.03 M pyruvate and 0.5 cc. of enzyme, fluoride (0.3 M), iodoacetate (0.003 M), arsenite (0.0003 M), benzoate (0.003 M), sulfathiazole (0.01 M), gramicidin (saturated solution), and azide (M) are without effect. Copper sulfate (0.0003 M) and capryl alcohol (saturated solution) cause complete inhibition. With cyanide (0.007 M), a 30 per cent inhibition was observed, but this effect may not be specific, since cyanide binds pyruvic acid as the cyanohydrin (20), thus lowering its effective concentration.

EXPERIMENTAL

Preparation—*Proteus vulgaris* (strain X-19) was grown in Roux bottles on tryptose-phosphate agar (Difco) for 16 hours at 37°. The bacteria were harvested by washing off the surface growth with small portions of 0.4 per cent saline, and then filtering the washings through several layers of muslin to remove agar particles. After hard packing in the centrifuge, the bacteria were twice resuspended and washed in 0.2 per cent saline. The final paste was stored at 4° in distilled water.

Disintegration was effected by one of two methods: (*a*) 25 cc. of a bacterial suspension containing about 25 mg. dry weight of bacteria per cc. were subjected for a period of 20 minutes in a 50 cc. conical flask to ultrasonic vibrations, generated by a crystal-controlled oscillator operating at 2000 volts and putting out about 600 watts. The piezoelectric crystal used was 1 inch in diameter and was ground to a frequency of 600 kc. To prevent overheating, the transformer oil which completely surrounded the crystal was circulated continuously by a centrifugal pump through copper coils in an ice water mixture. The temperature of the cell suspensions was never allowed to rise above 35°. (*b*) A thick cream (total volume about 35 cc.) was circulated through a Booth-Green mill (12) for about 1½ hours; practically complete disintegration took place.

The fresh juice (prepared by either method) was freed from intact cells and cellular débris by repeated centrifugation in a conical head centrifuge. It was then heated to 60° for 5 minutes at pH 7.0, chilled, and acidified with 10 per cent acetic acid to pH 4.3. The copious, white precipitate of protein was spun down at 1000 R.P.M. at 0°, the protein-free supernatant discarded, and the white pellet resuspended and washed twice in distilled water. The pellet was then resuspended in distilled water to which a few drops of 10 per cent sodium bicarbonate had been added. The final volume was usually one-fifth to one-eighth of the original volume of fresh juice. The resulting opalescent, somewhat viscous solution was dialyzed overnight at 4° against a large volume of 0.1 per cent saline or distilled water. This preparation

was then spun for 15 minutes at 13,000 R.P.M. in the high speed head of the refrigerated International centrifuge, No. 1, to free it of insoluble, inactive material. The resulting supernatant juice, used in all experiments described, was slightly turbid, gave no blank, and was completely inactive unless diphosphothiamine was added. Less than 1 γ of either inorganic phosphate or acid-hydrolyzable phosphate (7 minutes at 100° in 1 N sulfuric acid) was present per cc. of preparation. About 65 γ of organic phosphate per mg. of bacterial N was present.

Pyruvic acid oxidases of *Escherichia coli* (18) and *Lactobacillus delbrueckii* (1) were prepared by the methods described above. *Lactobacillus delbrueckii* was grown for 40 hours at 37° in a broth containing 0.8 per cent Bacto-liver (Difco), 0.5 per cent Bacto-yeast extract (Difco), 2 per cent glucose (U.S.P.), 0.8 neopeptone (Difco), and 5 gm. of precipitated calcium carbonate per liter of broth.

Reagents—Pyruvic acid was used in the form of the lithium salt prepared according to the method of Wendel (21). α-Ketobutyric acid and α-ketocaproic acid were prepared by the method of Schoenheimer and Ratner (22). Diphosphothiamine was kindly supplied by Merck and Company, Inc. Monophosphothiamine was prepared by the method of Lohmann and Schuster (23). We are indebted to Dr. D. W. Woolley for a sample of pyrithiamine hydrochloride, to Dr. H. Waelsch for a sample of phenylpyruvic acid, to Dr. H. McCoy of the University of Wisconsin for a culture of *Lactobacillus delbrueckii*, and to Dr. F. Lipmann for a generous sample of lithium acetyl phosphate.

Methods of Estimation—Pyruvic acid was determined by the bisulfite method of Clift and Cook (24), inorganic phosphate by the method of Fiske and Subbarow (25), and organic phosphate by the method of King (26). Acetyl phosphate was estimated by the hydroxamic acid-ferric chloride method of Lipmann (27). Thiamine was determined by the method of Urban and Goldman (28), the Beckman spectrophotometer being employed to measure the light absorption of thiochrome at 368 mμ. Diphosphothiamine, before being estimated as thiochrome, was first dephosphorylated to thiamine by the use of polidase.[6] 1 cc. of a 5 per cent solution of polidase was allowed to react with the diphosphoric ester at pH 4.0 at 37° for at least 1 hour.

Products of Reaction—In large scale reaction mixtures volatile acid was estimated by the method of Friedemann (29). The distillate was titrated with 0.02 N NaOH with phenolphthalein as indicator. Prior to titrating, the distillate was freed of CO_2 by rapid aeration with CO_2-free N_2.

In a typical manometric experiment, the enzymatic oxidation of 33.7

[6] Polidase is a commercial mold preparation which contains an active phosphatase and is obtainable from the Schwarz Laboratory, Inc., New York.

micromoles of pyruvic acid required 33.2 microatoms of oxygen and yielded 31.3 micromoles of CO_2. The stoichiometric relationship between the disappearance of pyruvic acid and the formation of acetic acid was determined as follows: 1000 micromoles of lithium pyruvate were incubated with the complete enzyme system for 5 hours at 37°. 800 micromoles of pyruvic acid disappeared with the formation of 816 micromoles of a volatile acid which was identified as acetic acid by the formation of a blue color with lanthanum nitrate (30), by the distribution constant (29) between ether and water (85.4; 86.4 for control), and by the Duclaux constant (31) (6.8 to 7.2; 6.8 to 7.4 for control).

SUMMARY

Proteus vulgaris contains an enzyme which specifically catalyzes the oxidation of pyruvic acid to acetic acid and CO_2. For each molecule of pyruvic acid oxidized, 1 atom of oxygen is consumed and 1 molecule of acetic acid and of CO_2 are formed. The enzyme appears to be a complex consisting of (1) a specific protein, (2) diphosphothiamine, and (3) a bivalent metal, probably magnesium. As prepared, the enzyme is partially dissociated and requires the addition of both diphosphothiamine and an appropriate metal to attain maximum activity.

The intact bacterial cell contains a phosphatase which can split diphosphothiamine to thiamine. This phosphatase, although absent in the final preparation of pyruvic oxidase, may well account for the apparent dissociation of diphosphothiamine from the enzyme.

The pyruvic oxidases of *Proteus vulgaris* and *Escherichia coli* do not appear to require inorganic phosphate for activity. Moreover, in the presence of 0.1 M phosphate, acetyl phosphate is not formed as an intermediary. Pyrophosphate in 3.4×10^{-3} M concentration inhibits the enzyme activity but this inhibition is reversed by 0.7×10^{-3} M diphosphothiamine.

The general properties of the enzyme are described. The enzyme is associated with macro particles which can be effectively sedimented in strong gravitational fields.

The author is greatly indebted to Dr. D. E. Green for suggesting this problem, and for the guidance and encouragement he has given throughout the course of this research. He also wishes to express his thanks to Dr. S. Ratner and Dr. L. F. Leloir for much advice and assistance.

SUMMARY

1. Lipmann, F., in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, **7**, 248 (1939).
2. Koepsell, H. J., and Johnson, M. J., *J. Biol. Chem.*, **145**, 379 (1942).
3. Kalnitsky, G., and Werkman, C. H., *Arch. Biochem.*, **2**, 113 (1943)

4. Utter, M. F., and Werkman, C. H., *Arch. Biochem.*, **5**, 413 (1944).
5. Silverman, M., and Werkman, C. H., *J. Biol. Chem.*, **138**, 35 (1941).
6. Lipmann, F., in Nord, F. F., and Werkman, C. H., Advances in enzymology and related subjects, New York, **1**, 99 (1941).
7. Lipmann, F., *J. Biol. Chem.*, **155**, 55 (1944).
8. Kalckar, H. M., *Biol. Rev. Cambridge Phil. Soc.*, **17**, 28 (1942).
9. Green, D. E., Herbert, D., and Subrahmanyan, V., *J. Biol. Chem.*, **138**, 327 (1941).
10. Green, D. E., Westerfeld, W. W., Vennesland, B., and Knox, W. E., *J. Biol. Chem.*, **145**, 69 (1942).
11. Stumpf, P. K., and Green, D. E., *J. Biol. Chem.*, **153**, 387 (1944).
12. Booth, V. H., and Green, D. E., *Biochem. J.*, **32**, 855 (1938).
13. Keilin, D., *Proc. Roy. Soc. London, Series B*, **121**, 165 (1936).
14. Warburg, O., and Christian, W., *Biochem. Z.*, **298**, 368 (1938).
15. Theorell, H., *Biochem. Z.*, **278**, 263 (1935).
16. Feigl, F., in Spot tests, New York, 142 (1939).
17. Buchanan, R. E., and Fulmer, E. I., Physiology and biochemistry of bacteria, **1**, 69 (1928).
18. Still, J. L., *Biochem. J.*, **35**, 380 (1941).
19. Müller, O. H., and Baumberger, J. P., *J. Am. Chem. Soc.*, **61**, 590 (1939).
20. Green, D. E., and Williamson, S., *Biochem. J.*, **31**, 617 (1937).
21. Wendel, W. B., *J. Biol. Chem.*, **94**, 717 (1931–32).
22. Schoenheimer, R., and Ratner, S., *J. Biol. Chem.*, **127**, 301 (1939).
23. Lohmann, K., and Schuster, P., *Biochem. Z.*, **294**, 188 (1937).
24. Clift, F. P., and Cook, R. P., *Biochem. J.*, **26**, 1788 (1932).
25. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
26. King, E. J., *Biochem. J.*, **26**, 292 (1932).
27. Lipmann, F., *J. Biol. Chem.*, **159**, 21 (1945).
28. Urban, F., and Goldman, M. L., *J. Biol. Chem.*, **152**, 329 (1944).
29. Friedemann, T. E., *J. Biol. Chem.*, **123**, 161 (1938).
30. Krüger, D., and Tschirch, E., *Mikrochemie*, **8**, 218 (1930).
31. Shriner, R. L., and Fuson, R. C., in The systematic identification of organic compounds, New York, 2nd edition, 120 (1940).

THE DETERMINATION OF 2,2-BIS(*p*-CHLOROPHENYL)-1,1,1-TRICHLOROETHANE (DDT) IN ORGANS AND BODY FLUIDS AFTER ORAL ADMINISTRATION

By HENRY A. STIFF, Jr.,* and JULIO C. CASTILLO

(From the Chemistry Section, Antilles Department Medical Laboratory, United States Army)

(Received for publication, April 23, 1945)

The toxicity of the now widely used insecticide 2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane (DDT) indicates the necessity for studies on the presence of this substance in the organs and body fluids after oral administration. Smith and Stohlman (1), assuming that the compound is absorbed unchanged from the alimentary system, attempted such studies, basing their determinations on the organically bound chlorine content of the biological material under examination.

In a recent publication (2) the authors described a colorimetric method for the determination of DDT based on the red color formed when this substance is heated with xanthydrol and potassium hydroxide in an anhydrous pyridine solution. The reaction was shown to be sensitive to 10 γ and to detect small differences in concentrations within a range of from 10 to 200 γ.

The present communication presents a comparison of the results of the xanthydrol-KOH-pyridine method of the authors and the sodium reduction method of Smith and Stohlman when applied to the determination of DDT in tissues and body fluids of rabbits after the oral administration of toxic

EXPERIMENTAL

Dosage and Collection of Specimens—Four male rabbits, weighing about 2 kilos each, were deprived of food overnight and then given 0.55 gm. of DDT per kilo in solution in olive oil by stomach tube. Food and water were again supplied and made available to the animals throughout the experiment. Blood samples were taken from the ear vein of each rabbit 2, 4, 8, and 24 hours after dosing; the samples from the four rabbits were pooled according to the time of collection, making a total of four blood samples collected at the intervals specified. Urine was collected by catheterization at frequent intervals during the 48 hour period and all samples pooled. All feces were collected during the period of the experiment. At the end of 48 hours the animals were killed by exsanguina-

* Captain, Sanitary Corps, Army of the United States.

tion from the carotid artery. The blood was pooled as the 48 hour sample. The livers, kidneys, and bile from the animals were removed for analysis.

Extraction of Organic Chlorides and DDT—The method of extraction of organic chlorides and DDT from the fluids and tissues was essentially the same as that used by Smith and Stohlman. Portions of blood, liver, kidney, bile, and feces were macerated with an equal weight of anhydrous sodium sulfate, dried in an oven at 90°, ground to a fine powder, and extracted in a Soxhlet apparatus with acetone. The urine was extracted with ether after it had been acidified with acetic acid.

Determination of Organically Bound Chlorine—A portion of the acetone extract from each fluid and tissue was evaporated to dryness on the steam bath and the organically bound chlorine determined by the method of Smith and Stohlman. That is, the residue from the evaporated acetone extract was dissolved in hot absolute alcohol, treated with metallic sodium, made acid with nitric acid, decolorized with chloride-free charcoal, and the chloride determined by the Volhard method with M/35.46 silver nitrate in 10 per cent nitric acid and M/35.46 potassium thiocyanate with ammonium ferric sulfate as the indicator.

Determination of DDT—The remainder of the acetone extract, in each case, was evaporated to dryness, the residue decolorized by heating on the steam bath with a few ml. of 30 per cent hydrogen peroxide, and again evaporated to dryness. Any fats extracted were converted to soaps by treating the residue with alcoholic potassium hydroxide on the steam bath. The dry residue was then macerated with anhydrous sodium sulfate and extracted with ether which was filtered into a volumetric flask and made up to volume.

Portions of this ether solution were pipetted into test-tubes (16 × 150 mm.) and evaporated to dryness. To each tube were then added 2 ml. of the xanthydrol-KOH-pyridine reagent (2). Control tubes containing known amounts of DDT and a blank tube containing only the reagent were set up in the same manner. All tubes were placed in an oil bath at 120° for 8 minutes and then cooled by immersion in water for 1 minute. The contents of the tubes were then diluted with 4 ml. of pure pyridine, mixed, and transferred to colorimeter tubes.

A Hellige-Diller photoelectric colorimeter, model 400, equipped with a green filter (520 mμ) was used; the galvanometer was set at zero with a blank and readings on all tubes were taken. DDT was calculated from a standard color curve, the control tubes being used as checks.

Recovery of Added DDT by Xanthydrol-KOH-Pyridine Method—To portions of the same organs and fluids analyzed above, DDT was added in amounts comparable to that calculated from the organically bound chlorine content as determined by the sodium reduction method. These organs and

fluids were extracted as described and the DDT determined on the extract by the xanthydrol-KOH-pyridine method. In the recovery experiments all blood samples were pooled and treated as one sample.

TABLE I

DDT As Calculated from Organically Bound Chlorine Content and As Determined by Xanthydrol-KOH-Pyridine Method in Organs and Body Fluids of Rabbits after Oral Administration of 0.55 Gm. of DDT per Kilo

Body fluid or tissue	Hrs. after dosing	Sodium reduction method: Chlorine (organically bound)	Sodium reduction method: Calculated DDT	DDT determined by xanthydrol-KOH-pyridine method
		mg. per 100 ml. or gm.	*mg. per 100 ml. or gm.*	*mg. per 100 ml. or gm.*
Blood	2	3.4	6.8	0.0
"	4	4.4	8.8	0.0
"	8	3.6	7.2	0.0
"	24	3.9	7.8	0.0
"	48	4.9	9.8	0.0
Liver	48	7.5	15.0	0.0
Bile	48	27.5	55.0	0.0
Kidney	48	8.6	17.2	0.0
Urine	2–48	4.7	9.4	0.0
Feces	2–48	308.7	617.4	560.0

TABLE II

Recovery of Added DDT from Organs and Body Fluid by Xanthydrol-KOH-Pyridine Method

Body fluid or tissue	DDT added	DDT found	Recovery
	mg. per 100 ml. or gm.	*mg. per 100 ml. or gm.*	*per cent*
Blood	8.0	6.4	80
Liver	15.0	12.1	81
Bile	50.0	36.5	73
Kidney	17.0	16.0	94
Urine	10.0	8.5	85

Results

Table I gives the results of the two methods. DDT is calculated on the basis of the theoretical 50 per cent chlorine content.

Table II gives the results of the recovery experiments on added DDT by the xanthydrol-KOH-pyridine reaction.

DISCUSSION

From the data presented it appears that DDT when administered orally is not absorbed in its unchanged state, but that other organic compounds

of chlorine are formed which are assimilated and can be detected in the organs and body fluids. That these organic chlorides do not normally appear in the body fluids and tissues of animals was shown by Smith and Stohlman and checked by the authors. It seems likely that the transformation of DDT to other organic chlorides takes place in the alimentary system and that only these organic chlorides are absorbed.[1] Further studies are in progress at present on this aspect of the problem.

SUMMARY

The compound 2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane was not detectable in the organs or body fluids of rabbits by the xanthydrol-KOH-pyridine method when toxic doses of this substance were administered orally to these animals.

The presence of organic chlorides in these organs and body fluids after oral administration of DDT was confirmed.

BIBLIOGRAPHY

1. Smith, M. I., and Stohlman, E. F., *Pub. Health Rep., U. S. P. H. S.*, **59**, 984 (1944).
2. Stiff, H. A., and Castillo, J. C., *Science*, **101**, 440 (1945).
3. White, W. C., and Sweeney, T. R., *Pub. Health Rep., U. S. P. H. S.*, **60**, 66 (1945).

[1] Since this work was completed, White and Sweeney (3) by use of an x-ray powder diffraction method have identified di(*p*-chlorophenyl)acetic acid in the urine of rabbits given DDT by oral administration. They succeeded in isolating this substance from the urine and gave methods for preparing it from DDT. These investigators concluded from their experiments that DDT in its unchanged form, if present in the urine at all, is present in amounts less than 5 mg. per 100 ml. The compound di(*p*-chlorophenyl)acetic acid prepared from DDT by the method of White and Sweeney does not give the xanthydrol-KOH-pyridine reaction. This serves to confirm our conclusion that DDT is not present as such in the organs and body fluids after oral administration but is present in the form of a metabolite containing organically bound chlorine.

ENZYMATIC OXIDATION OF GLUTATHIONE*

By STANLEY R. AMES AND C. A. ELVEHJEM

(*From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison*)

(Received for publication, April 12, 1945)

The oxidation of reduced glutathione was first studied by Hopkins (1), who showed that this substance in a neutral aqueous solution rapidly absorbed oxygen, becoming converted to the oxidized or disulfide form. In the presence of animal tissue preparations, this disulfide form was rapidly reduced to the sulfhydryl form. Hopkins and Dixon (2) showed that this reduction was due in part to factors which are not destroyed by heating the tissue to 100°. It was postulated that the reduction of the disulfide group by such "thermostable preparations" was due to "fixed —SH groups;" *i.e.*, sulfhydryl groups which were not soluble in water and which were unable to react directly with oxygen. The "thermostable system" was later studied in greater detail by Hopkins (3), who showed that the amount of oxygen taken up by the system amounted to several times the equivalent of the —SH groups originally present as glutathione. The extra oxygen taken up was accounted for by oxidation of the proteins present.

Harrison (4) showed that the apparent autoxidation of glutathione was due to traces of catalytic metals present as impurities. He showed that in absolutely metal-free solutions glutathione was oxidized only very slowly and that on the addition of a trace of a catalytic metal such as copper, an immediate and rapid oxidation resulted.

These early investigations were all done with preparations of glutathione which were not crystalline and which contained considerable impurities. Hopkins (5) reinvestigated this impure substance and succeeded in finding a method for the isolation of pure crystalline glutathione. Meldrum and Dixon (6), using this pure preparation of Hopkins', reinvestigated the properties of glutathione and found significant differences in behavior from the impure preparations. They found that solutions of crystalline glutathione are not appreciably autoxidizable even on the addition of iron. They attributed the autoxidation of glutathione to the presence of two cofactors, present in traces as impurities in the early glutathione preparations; namely, iron (or copper) and some substance able to form cata-

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. These studies were aided by a grant from the Rockefeller Foundation.

lytically active complexes with metals. Crystalline glutathione, unlike the earlier preparations, produced no oxygen uptake when added to "thermostable tissue preparations." When the disulfide form was added to the tissue, it was reduced but no oxygen uptake resulted. The oxidation of glutathione was also inhibited by fresh washed muscle. They showed that free cysteine closely resembled the properties of the catalytic impurity in the glutathione, but that the catalytic substance was probably not cysteine itself.

Keilin (7) showed that cysteine is very rapidly oxidized by the cytochrome-cytochrome oxidase system, with uptake of oxygen, and it might be expected that glutathione would be oxidized by the same system. Keilin stated, however, in the article by Meldrum and Dixon (6), that "crystalline glutathione is not oxidized by this system" and that it "reduces cytochrome only with extreme slowness." On the other hand, Stotz *et al.* (8) reported that "glutathione did not require the presence of ascorbic acid to be oxidized rapidly by the cytochrome-indophenol oxidase system" but did not amplify this statement or give any experimental data.

Lyman and Barron (9) could not confirm the reports (6, 10) that hematin was not an effective catalyst for the oxidation of crystalline glutathione. On the contrary, they indicated that glutathione solutions were readily oxidized in the presence of copper, hemin, or hemochromogens.

In this contribution, the oxidation of glutathione is investigated in greater detail. We will attempt to show that glutathione is oxidized by an enzymatic system involving cytochrome *c*. This observation throws considerable light on the results obtained by previous workers and shows how glutathione may function in biological oxidation-reduction systems.

Methods

White mice of an inbred Swiss strain were used and, after weaning, were maintained on stock ration[1] and water *ad libitum* plus occasional greens. A tissue homogenate of mouse kidney was prepared by the technique of Potter and Elvehjem (11), modified as previously described (12, 13). The unbroken cells and heavier cell fragments were removed by centrifugation at about 2500 r.p.m. in an angle head centrifuge for 5 minutes and the resulting supernatant is referred to as a "cell-free preparation." These cell-free preparations were used immediately in order to minimize enzyme inactivation occurring in the very dilute protein solutions (14).

A conventional Warburg apparatus at 37° was used in all experimental and analytical work. The pH was determined in all cases with a Beckman

[1] B-B Laboratory Rabbit Diet, Maritime Milling Company, Inc., Buffalo, New York.

pH meter (glass electrode). A summary of the components of the final reaction mixture is as follows: 0.4 ml. of 0.25 M potassium phosphate buffer (pH 7.6), 0.1 ml. of 1.4×10^{-4} M cytochrome *c*, the desired amount of cell-free preparation, solution of glutathione, and redistilled water to make 3.0 ml. The gas phase was air, and 0.2 ml. of 10 per cent KOH and a small strip of filter paper were placed in the center well to absorb CO_2. The pH of this final reaction mixture was 7.6, as determined electrometrically. The glutathione solution was always placed in a side arm and added to the mixture of the other components after equilibration of the flask contents had taken place.

Pure crystalline glutathione was obtained from the Eastman Kodak Company and was not purified further. The autoxidation of this preparation in glass-redistilled water was comparable with that observed by Meldrum and Dixon (6) for their purest preparations, and further purification was deemed unnecessary. Glutathione solutions were prepared in glass-redistilled water and were neutralized just before addition to the side arm of the reaction vessel. For comparative purposes a sample of crystalline glutathione from the B. L. Lemke Company was secured and was shown to be enzymatically oxidized at the same rate as the Eastman Kodak Company product.

The cytochrome *c* was prepared in these laboratories from beef heart by the method of Keilin and Hartree (15) and had been dialyzed against glass-redistilled water. Two samples of cytochrome *c*, which had been isolated in another laboratory,[2] were compared at similar levels with our preparation and shown to give identical results.

EXPERIMENTAL

During the course of some experiments in which attempts were made to place glutathione in carbohydrate metabolism as a coenzyme, it was observed that, in the presence of numerous added cofactors, tissue homogenates had the ability to oxidize reduced glutathione rapidly and completely. Determination of the particular components of the system which would oxidize reduced glutathione resulted in the isolation of a system in which cytochrome *c* was a necessary component. The results of a preliminary experiment plotted in Fig. 1 serve to illustrate this point. Curve I shows the slow autoxidation of reduced glutathione in neutral aqueous solution containing only phosphate buffer. The rate of oxygen uptake was comparable with that observed by previous investigators (6) for pure crystalline glutathione. Cytochrome *c* alone had no effect on this low

[2] Appreciation is expressed to Professor Van R. Potter of the McArdle Memorial Laboratory who kindly placed samples of two different preparations of cytochrome *c* at our disposal.

rate of oxygen uptake, showing that it did not affect the autoxidation of glutathione as was reported for hemin and hemochromogens by Lyman and Barron (9). Both Curves I and II are characterized by a considerable induction period before the slow autoxidation starts. Curve III shows the effect of adding a cell-free preparation of mouse kidney with no addition of cytochrome *c*. After an initial induction period of 40 to 50 minutes, during

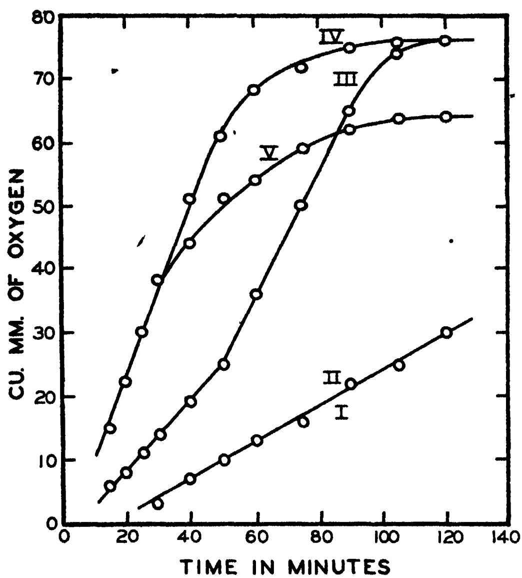


FIG. 1. Effect of cytochrome *c*, copper, and tissue preparations on glutathione oxidation. Curve I, buffer only; Curve II, buffer and cytochrome *c* (identical with Curve I); Curve III, buffer and cell-free tissue preparation; Curve IV, buffer, cytochrome *c*, and cell-free tissue preparation; Curve V, buffer and cupric ion. Reduced glutathione was present in all cases at a level of 3 mg. per flask (equivalent to a total oxygen uptake of 64 c.mm.). Other final concentrations were buffer, M/30 potassium phosphate (pH 7.6); cytochrome *c* 4.7×10^{-6} M; cell-free tissue preparation representing 20 mg. wet weight of mouse kidney (dry weight of about 4 mg.); and cupric sulfate 10^{-4} M. Redistilled water was added to make a total volume of 3.0 ml. The tissue preparation was added, the flasks equilibrated, and the glutathione solution was added at zero time from the side arm.

which the rate was more rapid than when no tissue was added, the rate increased abruptly and was linear with time until the reduced glutathione was exhausted. When both cytochrome *c* and cell-free preparation were added in amounts corresponding to Curves II and III, respectively, an immediate rapid uptake was observed which was linear with time until the substrate was exhausted. The slope of this curve is the same as that plotted in Curve V, which represents the copper-catalyzed autoxidation of gluta-

thione. Note that the maximum value of oxygen uptake is greater when tissue is present than in its absence. The extra oxygen was probably due to either oxidation of tissue protein or metabolism of miscellaneous substrates in the cell-free extract.

From this experiment it was evident that cytochrome *c* was functioning with some enzyme in the tissue, by definition cytochrome oxidase, to oxidize reduced glutathione. Cytochrome *c* may be directly concerned in the oxidation of reduced glutathione or it may be acting in a system metabolizing some intermediate which itself is oxidizing glutathione.

When iron was added as ferric ion, glutathione was not autoxidized more rapidly, thus confirming the previous observations of Meldrum and Dixon (6). This renders invalid the possibility that cytochrome *c* is acting by means of the catalytic action of iron liberated from it by action of the tissue.

It has been observed (6) that the complex formed between cysteine and copper has the property of catalyzing the oxidation of reduced glutathione by oxygen. The possibility that cytochrome *c* is acting by catalyzing the hydrolysis of glutathione to yield cysteine, which would unite with available copper to catalyze the oxidation of glutathione, has been considered. This mechanism has been discarded, since it would imply that cytochrome *c* was functioning as an activator of a proteolytic enzyme, a concept not supported by the observed data.

Tissue Concentration—An investigation of the glutathione oxidase system was undertaken to see whether the rate was proportional to the concentration of the specific enzyme. The results plotted in Fig. 2 show that the rate is a function of the enzyme concentration under the given conditions only when 2 mg. or less of tissue on a wet weight basis are added. An increase in tissue level above this value exhibited no increase in the rate of oxidation, indicating that some factor other than enzyme concentration was limiting (in this case, probably cytochrome *c*).

The maximum oxygen uptake at all tissue levels below 2 mg. could be accounted for by the amount of reduced glutathione added. Generally when 3 mg. of glutathione were added per flask, there was no further oxygen uptake after 5 hours, even when no tissue preparation was added. However, when more than 2 mg. of tissue were added, the maximum oxygen uptake was considerably in excess of that which could be accounted for by the oxidation of added glutathione. This was probably due either to an oxidation of the free —SH groups in the protein as proposed by Hopkins (5) or perhaps to an oxidation of substrates previously shown to be present in tissue homogenates (12).

This system was active at very low levels of tissue concentration. The lowest level plotted indicates considerable increase in rate over that

obtained with no tissue and represents a concentration of about 0.02 mg. dry weight of tissue per flask. The extremely low tissue levels in which this system is active tend to eliminate the possibility that the oxidation of glutathione proceeds via some intermediate substrate present in the

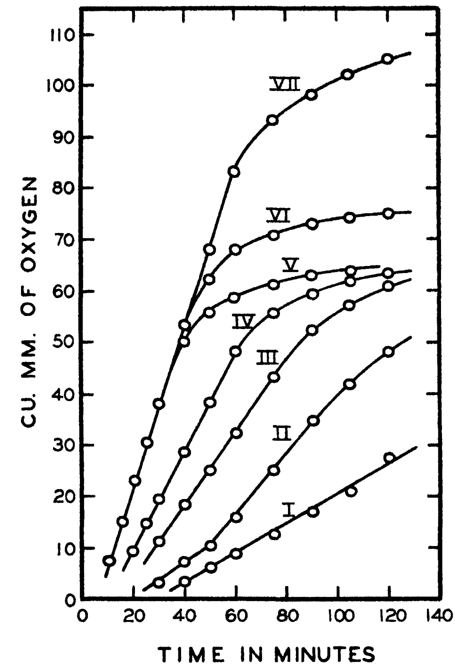


FIG. 2. Effect of tissue level on glutathione oxidation with added cytochrome *c*. Curve I, buffer only or buffer and 4.7×10^{-6} M cytochrome *c*, 3 mg. of glutathione; Curve II, buffer, 3 mg. of glutathione, 4.7×10^{-6} M cytochrome *c*, and cell-free tissue preparation representing 0.1 mg. wet weight of mouse kidney (dry weight of about 0.02 mg.); Curve III, same as Curve II, except 0.4 mg. of the above tissue preparation (wet weight); Curve IV, same as Curve II, except 1.0 mg. of tissue; Curve V, same as Curve II, except 2.0 mg. of tissue; Curve VI, same as Curve II, except 20.0 mg. of tissue; Curve VII, same as Curve II, except 100 mg. of tissue. Conditions not specified are the same as summarized in the legend of Fig. 1.

homogenate which is oxidized by the cytochrome *c*-cytochrome oxidase system.

When the Q_{O_2} values of the curves in Fig. 2 were plotted against tissue concentration, an inverse dilution effect resulted; that is, the Q_{O_2} was higher at lower tissue concentrations than at higher tissue concentrations. This effect had previously been noted by Schneider and Potter (16) for the cytochrome oxidase system with ascorbic acid as the substrate. They considered the uptake at zero tissue concentration (obtained by extra-

polation of values obtained at three or more tissue concentrations) to be a measure of the substrate autoxidation and subtracted this value from the uptake at all tissue levels. When this was done with the present system, a value was obtained for the uptake at zero tissue concentration which was considerably in excess of the value obtained experimentally with no tissue present. This perhaps indicates that the inverse dilution effect still observed after subtracting the experimentally obtained autoxidation at zero tissue concentration is due to secondary factors as yet not evaluated.

Cytochrome c Concentration—Since the effect of tissue level on the oxidation of glutathione had been determined, the effect of varying another factor in the system, cytochrome *c*, was investigated. A level of tissue was selected within the range in which tissue concentration was proportional to the rate of oxygen uptake. In this range it had been previously assumed that the concentration of cytochrome *c* was not limiting and this assumption needed confirmation. The amount of added cytochrome *c* per flask varied from 2.3×10^{-5} M to 4.7×10^{-10} M, as shown in Fig. 3. An increase in Q_{O_2} with an increase in cytochrome *c* concentration over that previously used at this tissue level was not observed, and a decrease in Q_{O_2} was evident if the cytochrome *c* level were decreased. The lower levels of added cytochrome *c* exhibited no effect over the oxidation achieved by cell-free preparation alone, indicating that the cell-free preparation was probably adding enough cytochrome *c* to raise the level of cytochrome *c* to a concentration of about 10^{-8} M.

Curve VI of Fig. 3 indicates that the enzyme is saturated with cytochrome *c* at the level of 4.7×10^{-6} M when a cell-free preparation representing 1 mg. of mouse kidney homogenate is used. Schneider and Potter (16), with ascorbic acid as the substrate of their cytochrome oxidase assay system, reported a saturation level of 7×10^{-5} M cytochrome *c* for 5 mg. of fresh rat liver, a level which can be compared with the value recorded above.

Glutathione Concentration—In order to devise an assay method to determine the concentration of the glutathione oxidase system, the enzyme must be saturated with the substrate. The experiments plotted in Fig. 4 indicate the results obtained with several different amounts of glutathione added to a system in which both tissue and cytochrome *c* had previously been shown to be non-limiting. When both tissue and cytochrome *c* were present, the maximum oxygen uptake was a linear function of the amount of glutathione added but the rate of oxygen uptake, a direct function, asymptotically approached a maximum slope. In the absence of tissue, *i.e.*, autoxidation of glutathione in the presence of cytochrome *c*, the maximum oxygen uptake was again a linear function of the glutathione concentration. However, in contrast to the results obtained when tissue was added, the slope in this case was also a linear function of the substrate

added. At the same glutathione concentration, both curves attained the same maximum value, but the one representing only autoxidation did so much more slowly. A maximum Q_{O_2} of 418 was obtained when the glutathione concentration was 9×10^{-3} M, this value being corrected for the autoxidation of glutathione at zero tissue concentration, as determined

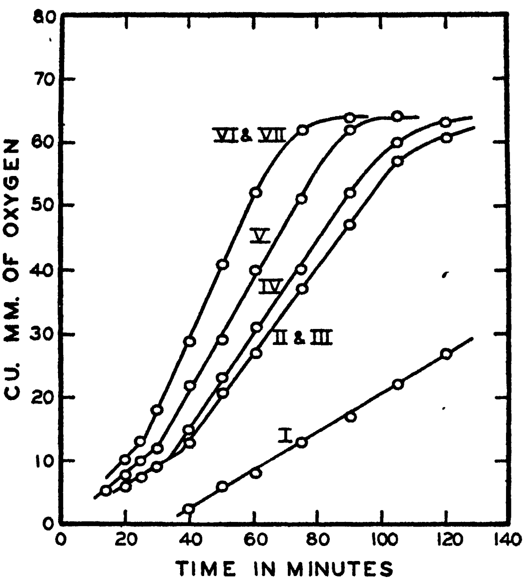


FIG. 3. Effect of added cytochrome *c* on enzymatic oxidation of glutathione. Curve I, buffer only or buffer and 4.7×10^{-6} M cytochrome *c*, 3 mg. of glutathione; Curve II, buffer, 3 mg. of glutathione, and cell-free tissue preparation representing 1.0 mg. wet weight of mouse kidney (dry weight of about 0.2 mg.), no cytochrome *c* added; Curve III, buffer, 3 mg. of glutathione, and tissue preparation as above; concentration of added cytochrome *c* in reaction mixture of either 4.7×10^{-10} M or 4.7×10^{-9} M (identical with Curve II); Curve IV, same as Curve III, except 4.7×10^{-8} M cytochrome *c*; Curve V, same as Curve III, except 4.7×10^{-7} M cytochrome *c*; Curve VI, same as Curve III, except 4.7×10^{-6} M cytochrome *c*; Curve VII, same as Curve III, except 2.3×10^{-5} M cytochrome *c* (identical with Curve VI). Conditions not specified are the same as summarized in the legend of Fig. 1.

experimentally. This Q_{O_2} value can be compared with the Q_{O_2} of 695 reported by Schneider and Potter (16) for the cytochrome oxidase system from rat kidney with ascorbic acid as substrate.

Inhibitors—If the oxidation of reduced glutathione were enzymatic in nature, the enzyme should be inactivated by heat. An experiment was performed in which a 10 per cent homogenate was heated to 85° for 5 minutes, then centrifuged and decanted, as previously described, to obtain a cell-free preparation. This preparation proved to be 100 per cent in-

activated,[3] indicating that some component of the system was destroyed at the temperature used.

A number of chemical inhibitors were tried, both to determine what substances inhibited the system and to ascertain if possible the nature of

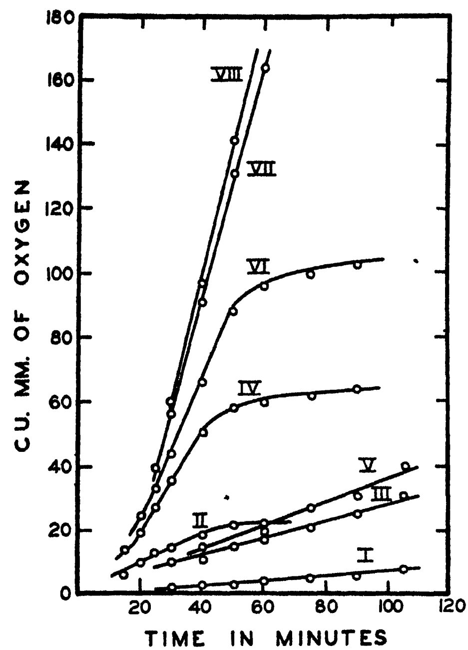


FIG. 4. Effect of glutathione concentration on its enzymatic and non-enzymatic oxidation. Curve I, buffer only or buffer and 4.7×10^{-6} M cytochrome *c*, glutathione concentration of 0.9×10^{-3} M (1 mg. per reaction flask); Curve II, buffer, cytochrome *c*, cell-free tissue preparation representing 2.0 mg. wet weight of mouse kidney (dry weight of about 0.4 mg.), glutathione as in Curve I; Curve III, same as Curve I, except glutathione concentration 2.7×10^{-3} M; Curve IV, same as Curve II, except glutathione concentration 2.7×10^{-3} M; Curve V, same as Curve I, except glutathione concentration 4.5×10^{-3} M; Curve VI, same as Curve II, except glutathione concentration 4.5×10^{-3} M; Curve VII, same as Curve II, except glutathione concentration 9×10^{-3} M; Curve VIII, same as Curve II, except glutathione concentration 18×10^{-3} M. Conditions not specified are the same as summarized in the legend of Fig. 1.

the enzyme concerned. The data of Table I indicate the approximate inhibitions obtained. Since there were numerous complicating secondary factors to be controlled in determining the degree of inhibition (12) and

[3] The components of the reaction mixture were the same as summarized in Table I, except that an amount of cell-free preparation representing 1 mg. of mouse kidney previously treated as described in the text was added.

since only a qualitative comparison was desired, only an approximate value of the per cent inhibition was recorded. Inspection of the data indicates that those substances which caused marked inhibition were those which are known to react with copper; namely, cyanide and diethyldithiocarbamate.

TABLE I

Effect of Inhibitors on Enzymatic Oxidation of Glutathione

Inhibitor	Concentration	Approximate per cent inhibition
	M	
Azide	0.002	4
Cyanide	0.002	100+
	0.0002	100
	0.00002	40
α,α'-Dipyridyl	0.002	40
Diethyldithiocarbamate	0.002	100
	0.0002	100
	0.00002	28
Hydroxylamine	0.002	8
Iodoacetate	0.002	100+
	0.0002	32
	0.00002	3
Thioglycolate	0.002	30

Constituents were added to the reaction flask as follows: 0.4 ml. of 0.25 M potassium phosphate buffer (pH 7.6), 0.1 ml. of 1.4×10^{-4} M cytochrome *c*, 0.3 ml. of a 10 mg. per ml. solution of reduced glutathione (neutralized with sodium hydroxide to pH 7.6) placed in side arm, solution of inhibitor (pH 7.6) to yield the concentration indicated, and redistilled water to make 2.8 ml. 0.2 ml. of a 1 per cent cell-free preparation of mouse kidney homogenate was added, the flask equilibrated, and the glutathione solution added from the side arm at zero time. The tissue was in contact with the inhibitor about 12 minutes before the glutathione was added.

In all the inhibitor studies, the KOH solution in the center well was eliminated to prevent changes in the concentration of such inhibitors as azide and cyanide. Suitable control flasks showed that the absence of the KOH had no effect on either the Q_{O_2} or the total oxygen uptake.

The degree of inhibition was calculated from Q_{O_2} values, each experiment having its set of controls. An inhibition of 100+ per cent indicates that not only the enzymatic oxidation of glutathione was inhibited but also the buffer-catalyzed nonenzymatic oxidation.

Iodoacetate, which also caused marked inhibition, was presumed to react with the sulfhydryl group of glutathione and so reduce the substrate concentration. On the other hand the "iron reagents" such as thioglycolate, α,α'-dipyridyl, and hydroxylamine did not exhibit marked inhibition of the glutathione oxidation system. Sodium azide, contrary to expectation, showed little or no inhibition when present in 0.002 M concentration.

The results of these preliminary inhibitor studies indicate that the glutathione oxidase system involves a heat-labile enzyme and that in all probability this enzyme is a copper-protein.

DISCUSSION

When Hopkins (1) discovered glutathione, he considered that he had isolated one of the catalysts of cellular oxidations. Even though it was relatively inert to molecular oxygen and therefore could not act in a terminal oxidase system, this concept continued until it was eventually overshadowed by Keilin's isolation of the cytochrome *c*-cytochrome oxidase system.

The rôle which has been ascribed to glutathione by Lyman and Barron (9) is to maintain the intensity level of reduction and in this manner to regulate the rate of reversible oxidation-reduction reactions. Barron and Singer (17–19) later modified this general concept by stating that glutathione functions by maintaining the activity of enzyme systems containing essential —SH groups in their protein moiety. This concept of glutathione action is rather limited and tends to ignore any possibility of its acting as an intermediate "coenzyme" in oxidation-reduction systems.

Hopkins (1) showed very early in his glutathione studies that oxidized glutathione was reduced by tissue preparations. Hopkins and Elliott (20), using crystalline glutathione, were able to repeat this earlier work. Mann (21) reported the reduction of glutathione during the oxidation of glucose in the presence of "glucose dehydrogenase," and Meldrum and Tarr (22) reported its reduction by hexose monophosphate in the presence of Warburg and Christian's "enzyme" and "coenzyme" systems. Potter (23) showed that glutathione exerted a marked effect on pyruvate oxidation in a reconstructed enzyme system with cell-free tissue extracts. It is evident that glutathione can be readily reduced by enzyme systems, but a specific method for its enzymatic oxidation by tissues has previously been lacking (24).

As a result of our observations that reduced glutathione (GSH) can be oxidized (GSSG) by a cell-free tissue preparation when cytochrome *c* is added, we can propose that glutathione is oxidized via the following mechanism.

$$\begin{array}{ccccc}
\mathrm{GSH} & & \text{cytochrome } c\ \mathrm{Fe}^{++} & & \mathrm{H_2O} \\
\uparrow\downarrow & \rightleftharpoons & \uparrow\downarrow & \rightleftharpoons & \uparrow\downarrow \\
\tfrac{1}{2}\mathrm{GSSG} & & \text{cytochrome } c\ \mathrm{Fe}^{+++} & & \tfrac{1}{2}\mathrm{O_2}
\end{array}$$

Except for the results of inhibitor studies, which indicate that a copper-containing enzyme is an integral component of this system, this mechanism is strongly supported by the observed data. Cytochrome oxidase is by

definition necessary to catalyze the oxidation of reduced cytochrome *c* by molecular oxygen and is generally considered to be an iron-protein enzyme. In addition cytochrome oxidase has been frequently cited as being strongly inhibited by azide (25), but this system showed little inhibition at a relatively high concentration of azide. The implications of this observation are not yet fully understood and on consideration of the strong supporting evidence the above mechanism is postulated to be correct.

In addition we suggest that glutathione can function as a coenzyme in an oxidation-reduction system. Mechanisms for both the oxidation and reduction of glutathione by enzymes have now been observed in tissue preparations. The additional oxygen uptake observed when more than 2 mg. of tissue are added to the reaction flask is readily explained by oxidation of the free —SH groups in the tissue protein. Oxidized glutathione is reduced by the free —SH groups and the reduced glutathione formed is reoxidized via the above mechanism. Glutathione is thus acting as a coenzyme in the system involving the oxidation by molecular oxygen of fixed sulfhydryl groups in the cell-free tissue preparation.

Whether a specific enzyme functions between glutathione and cytochrome *c* is a question for which preliminary data only have been obtained. The reduction of cytochrome *c* by reduced glutathione proceeds much more rapidly in the presence of cyanide-inhibited tissue homogenate than when no tissue is present. Cyanide-inhibited tissue homogenate will reduce cytochrome *c* in the absence of glutathione, but at a much slower rate and not as completely as when glutathione is present. These data may be interpreted as indicating the possibility of an enzyme catalyzing the reaction between oxidized cytochrome *c* and reduced glutathione; *i.e.*, glutathione dehydrogenase. In addition, comparison of this investigation with that of Keilin (7), who did not obtain any enzymatic oxidation, indicates certain probable differences. No experimental data were given to substantiate Keilin's conclusions, but presumably he used a semipurified indophenol oxidase preparation. In this work a tissue preparation containing many enzymes was used and herein may lie a difference; his preparations may not have contained a hypothetical enzyme functioning between glutathione and cytochrome *c*.

The oxidation of glutathione through the intermediary action of cytochrome *c* serves to explain the anomalous results of Hopkins and associates. The "thermostable tissue preparations" may have had free sulfhydryl groups present which would reduce any oxidized glutathione added, but the enzymatic systems for the oxidation of reduced glutathione may have been destroyed. Hence, with pure crystalline glutathione, oxidized glutathione would be reduced but no oxygen uptake would result. However, when impure glutathione was added to "thermostable preparations," enough

impurities (presumably cysteine) may have been present to form catalytically active complexes with copper as postulated by Meldrum and Dixon (6). Thus the glutathione would be oxidized and, on being simultaneously reduced by the free —SH groups in the tissue preparation, would take up oxygen until the reducing groups were all oxidized. Observations with washed muscle tissue can be explained in a similar fashion. Washing the tissue effectively would remove cytochrome *c*, so that even though the enzymes were probably active, no oxygen uptake would result on addition of pure glutathione. The free —SH groups, however, again would reduce the oxidized glutathione. Impure glutathione, by itself being catalytically oxidized, might function as an intermediate between the free —SH groups of the washed muscle preparation and molecular oxygen. If it were being simultaneously oxidized and reduced as described in this system, glutathione would be functioning as a coenzyme in the oxidation of fixed —SH groups by molecular oxygen.

SUMMARY

1. Reduced glutathione can be oxidized by an enzymatic system involving cytochrome *c*, which has been found in cell-free preparations of mouse kidney homogenates.

2. The effects of tissue concentration, cytochrome *c* concentration, and glutathione concentration on this system are reported.

3. The glutathione oxidase system is strongly inhibited by heat, cyanide, diethyldithiocarbamate, and iodoacetate, is weakly inhibited by α,α'-dipyridyl, and thioglycolate, and is not inhibited by azide and hydroxylamine.

4. Glutathione is postulated to act as a coenzyme in certain systems involving the oxidation by molecular oxygen of fixed sulfhydryl groups occurring in tissue preparations.

BIBLIOGRAPHY

1. Hopkins, F. G., *Biochem. J.*, **15**, 286 (1921).
2. Hopkins, F. G., and Dixon, M., *J. Biol. Chem.*, **54**, 7 (1922).
3. Hopkins, F. G., *Biochem. J.*, **19**, 787 (1925).
4. Harrison, D. G., *Biochem. J.*, **18**, 1009 (1924).
5. Hopkins, F. G., *J. Biol. Chem.*, **84**, 269 (1929).
6. Meldrum, N. U., and Dixon, M., *Biochem. J.*, **24**, 472 (1930).
7. Keilin, D., *Proc. Roy. Soc. London, Series B*, **106**, 418 (1930).
8. Stotz, E., Harrer, C. J., Schultze, M. O., and King, C. G., *J. Biol. Chem.*, **122**, 407 (1938).
9. Lyman, C. L., and Barron, E. S. G., *J. Biol. Chem.*, **121**, 275 (1937).
10. Voegtlin, C., Johnson, J. H., and Rosenthal, S. M., *Pub. Health Rep., U. S. P. H. S.*, **46**, 2234 (1931).
11. Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, **114**, 495 (1936).

12. Ames, S. R., and Elvehjem, C. A., *Arch. Biochem.*, **5**, 191 (1944).
13. Ames, S. R., and Elvehjem, C. A., *Proc. Soc. Exp. Biol. and Med.*, **57**, 109 (1944).
14. Ames, S. R., and Elvehjem, C. A., *Proc. Soc. Exp. Biol. and Med.*, **58**, 52 (1945).
15. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, **122**, 298 (1937).
16. Schneider, W. C., and Potter, V. R., *J. Biol. Chem.*, **149**, 217 (1943).
17. Barron, E. S. G., and Singer, T. P., *Science*, **97**, 356 (1943).
18. Barron, E. S. G., in Nord, F. F., and Werkman, C. H., Advances in enzymology and related subjects, New York, **3**, 176 (1943).
19. Singer, T. P., and Barron, E. S. G., *J. Biol. Chem.*, **157**, 241 (1945).
20. Hopkins, F. G., and Elliott, K. A. C., *Proc. Roy. Soc. London, Series B*, **109**, 58 (1931).
21. Mann, P. J. G., *Biochem. J.*, **26**, 785 (1932).
22. Meldrum, N. U., and Tarr, H. L. A., *Biochem. J.*, **29**, 108 (1935).
23. Potter, V. R., in Nord, F. F., and Werkman, C. H., Advances in enzymology and related subjects, New York, **4**, 237 (1944).
24. Ogston, F. J., and Green, D. E., *Biochem. J.*, **29**, 1983 (1935).
25. Respiratory enzymes, Minneapolis, 41 (1939).

LETTERS TO THE EDITORS

GUANINE AS A COENZYME IN ENZYMATIC TRANSPHOSPHORYLATION

Sirs:

It has been reported[1] that both dihydrocozymase and inorganic phosphate are necessary for the following reactions in rat muscle extracts.

(1) Glucose + adenosine triphosphate
→ glucose-6-phosphate + adenosine diphosphate

(2) Fructose-6-phosphate + adenosine triphosphate
→ fructose-1,6-diphosphate + adenosine diphosphate

The phosphate has now been found necessary for the formation of guanine from a precursor present in dialyzed extracts, by a reaction which is apparently analogous to the new type of phosphorolysis recently described by Kalckar.[2]

(3) Purine-riboside + phosphate ⇌ purine + ribose-1-phosphate

When aqueous extracts of acetone-dried or fresh rat muscle are dialyzed against saline for 10 to 20 hours, the addition of sodium phosphate (0.005 M) is sufficient for reactivation, since dihydrocozymase is not removed by this procedure. The reactivation by phosphate occurs after a lag period of 5 to 30 minutes (Fig. 1) and is completely prevented by iodoacetate (0.002 M).

After incubation of a dialyzed extract with phosphate, a substance can be isolated which causes reactivation of dialyzed extracts in the absence of inorganic phosphate. This substance, which can also be obtained from boiled muscle juice or by acid hydrolysis of ribo- or desoxyribonucleic acid, acts immediately, with no lag period (Fig. 1), and its effect is not abolished by iodoacetate (Fig. 2). Of the two purines freed on mild acid hydrolysis of nucleic acid, adenine was without effect, while 10^{-5} M guanine hydrochloride (Eastman) caused full reactivation (Fig. 2).

The active substance formed on incubation of a dialyzed extract with

[1] Price, W. H., and Colowick, S. P., *Federation Proc.*, 4, 100 (1945).
[2] Kalckar, H. M., *J. Biol. Chem.*, 158, 723 (1945).

phosphate is precipitated by the purine precipitant, $CuSO_4$-$NaHSO_3$. The activity of the precipitated material is that to be expected from its guanine content (Fig. 2), as determined either colorimetrically[3] or from light absorption measurements at 250 mμ. Phosphate is necessary for, and iodoacetate inhibits, the appearance of the active substance and also

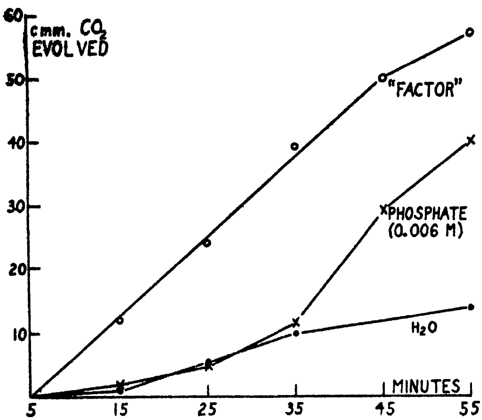


Fig. 1

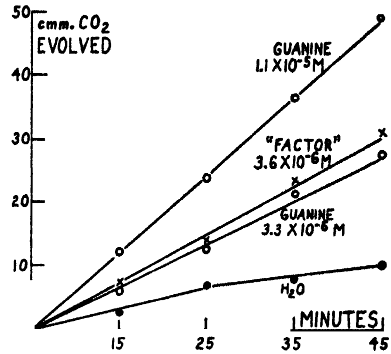


Fig. 2

Fig. 1. Effect of phosphate and "factor" on Reaction 1. Manometric measurement as described previously,[4] but without addition of iodoacetate. Additions marked on curves, tipped in from side arm at zero time. "Factor" prepared from boiled juice of rat muscle by concentrating supernatant fluid from precipitation of Ba salts with 80 per cent alcohol. The amount added was that obtained from 1 gm. of muscle.

Fig. 2. Effect of "factor" and guanine on Reaction 1. 0.002 M iodoacetate present. "Factor" prepared by incubation of dialyzed extract with phosphate, concentration of the deproteinized solution, and precipitation with $CuSO_4$-$NaHSO_3$ reagent. Concentration calculated from guanine estimation (see the text).

the appearance of the color test and absorption band characteristic of guanine.

It appears that both dihydrocozymase and guanine are essential coenzymes for Reactions 1 and 2.

It is a pleasure to thank Professor C. F. Cori for his suggestions during the course of this work.

Department of Pharmacology
Washington University School of Medicine
St. Louis

Sidney P. Colowick
Winston H. Price

Received for publication, May 31, 1945

[3] Hitchings, G. H., *J. Biol. Chem.*, **139**, 843 (1941).
[4] Colowick, S. P., and Price, W. H., *J. Biol. Chem.*, **157**, 415 (1945).

ISOLATION AND CRYSTALLIZATION OF *d*-GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE FROM RABBIT MUSCLE

Sirs:

The method for the preparation of this enzyme which is described below requires only a few steps, the yield is nearly quantitative, and after recrystallization the protein is homogeneous in the Tiselius apparatus. The muscles of one rabbit, killed by intravenous injection of amytal, are rapidly excised and passed twice through a meat grinder. All steps are carried out in a cold room at 5°. The ground muscles are extracted twice with 1 volume of 0.03 N KOH and filtered through gauze. The pH of the combined extract is between 6.7 and 7.0. Ammoniacal ammonium sulfate solution (20 to 25 cc. of ammonium hydroxide, sp. gr. 0.90, to 1 liter of saturated ammonium sulfate solution, pH about 8.4) is added to 52 per cent saturation. The precipitate is filtered off. To the clear filtrate are added 13 gm. of solid ammonium sulfate per 100 cc., which brings the saturation to 72 per cent (sp. gr. 1.188). The precipitate is filtered off. Crystals appear overnight in the originally clear filtrate and continue to form for several days. The crystals are arranged in rosettes made up of diamond-shaped plates which stand on edge. The protein can be recrystallized from 66 per cent saturated ammoniacal ammonium sulfate solution. The crystals then appear singly rather than in rosettes. The yield of crystals is very high. An example is given below.

590 gm. of rabbit muscles yielded 950 cc. of extract containing 16.5 gm. of protein. After the precipitates which formed at 52 and 72 per cent saturation had been removed, the filtrate measured 1900 cc. and contained 3.27 gm. of protein. Of this amount 1.14 gm. were obtained as the crystalline enzyme. This is equivalent to 7 per cent of the extracted proteins or to 0.19 gm. per 100 gm. of muscle.

Activity measurements were carried out as described by Warburg and Christian[1] for the crystalline yeast enzyme, except that cysteine was added; a reducing agent is necessary for full activity of the muscle enzyme. In the following experiment the composition of the reaction mixture (in moles per cc.) was as follows: cozymase and *dl*-glyceraldehyde phosphate (as the *d* form) each 2.5×10^{-7}, sodium arsenate 5.7×10^{-6}, sodium pyrophosphate 3×10^{-5}, cysteine 3.6×10^{-6}, enzyme protein (5 times recrystallized) 0.003 mg. per cc., pH 8.5, temperature 25°. Readings were made in a quartz cell in the Beckman spectrophotometer at 340 mμ against a cell set at 100 per cent transmission which contained all the

[1] Warburg, O., and Christian, W., *Biochem. Z.*, **303**, 40 (1939–40).

reactants except the enzyme. The reaction was started by the addition of triose phosphate. The time required for half reaction, calculated from $1/K\,a$, where K is the bimolecular rate constant and a is the initial concentration, was 2.3 minutes. Because of the need of a reducing agent the exact conditions for optimal activity of *d*-glyceraldehyde dehydrogenase from muscle have not yet been established. The values for the rate constant so far obtained are of the same order of magnitude as those given for the yeast enzyme.[1]

We wish to thank Dr. H. O. L. Fischer for a generous gift of *dl*-glyceraldehyde phosphate prepared by Dr. Lardy.

Department of Pharmacology
Washington University School of Medicine
St. Louis

Gerty T. Cori
Milton W. Slein
Carl F. Cori

Received for publication, June 18, 1945

THE UTILIZATION OF GLYCINE FOR THE SYNTHESIS OF A PORPHYRIN

Sirs:

It is known that porphyrin can be synthesized by the animal. The nature of the precursors employed for the formation of the protoporphyrin of hemoglobin has been the subject of much speculation.[1] This report presents evidence for the direct utilization of glycine for the formation of the pyrrole rings of protoporphyrin in humans.

One of us consumed, over a period of 3 days, 66 gm. of glycine containing 32.4 atom per cent excess N^{15} in addition to the usual diet. Samples of hemin were prepared from blood samples taken after 4, 18, 77, 86, and 99 days from the start of the experiment; the N^{15} concentration of these was 0.134, 0.422, 0.466, 0.460, and 0.445 atom per cent excess respectively. By interpolation it is estimated that the isotope concentration in the porphyrin reached a maximum value of 0.52 atom per cent excess 30 days after the start of the experiment. At this time the isotope concentration of the plasma proteins had declined from a maximum value of 0.39 atom per cent excess N^{15} attained on the 4th day of the experiment to 0.13 atom per cent excess.

From the isotope concentrations of the hemin of the 77th, 86th, and 99th days we estimate that the average life time of the protoporphyrin is more than 100 days. A more precise value for the average life time can be obtained only after many months. From the approximate value of the average life time it can be estimated that in 18 days about 20 per cent of the porphyrin was newly synthesized. Since the porphyrin at this time contained 0.42 atom per cent excess N^{15}, the newly formed porphyrin which contains the N^{15} must have an average N^{15} concentration of 4.1 atom per cent excess; its nitrogenous precursor must therefore have had the same average N^{15} concentration. The only substance that could have had an average value of about 2 per cent N^{15} during the first 18 days is the glycine of dietary origin. Previous experiments from this laboratory in which leucine[2] and ammonia[3] labeled by N^{15} were fed to rats gave no indication that these substances were directly concerned with porphyrin synthesis.

[1] Abderhalden, E., Lehrbuch der physiologischen Chemie, Berlin and Vienna, 744 (1909). Lusk, G., The elements of the science of nutrition, Philadelphia and London, 4th edition, 244 (1928).

[2] Schoenheimer, R., Ratner, S., and Rittenberg, D., *J. Biol. Chem.*, **130**, 703 (1939).

[3] Rittenberg, D., Schoenheimer, R., and Keston, A. S., *J. Biol. Chem.*, **128**, 603 (1939).

It has recently been reported by Bloch and Rittenberg that acetic acid participates in the synthesis of protoporphyrin.[4] It was suggested that a derivative of acetic acid may be utilized in a condensation resembling the Knorr synthesis for pyrroles. It may be concluded that the protoporphyrin is synthesized *in vivo* from glycine and either acetic acid or some compound closely related to it. An analogous *in vitro* reaction is now known to occur; Fischer has recently found that a positive color test for pyrroles could be obtained after condensing formylacetone with glycine.[5]

Department of Biochemistry
College of Physicians and Surgeons
Columbia University
New York

DAVID SHEMIN
D. RITTENBERG

Received for publication, June 18, 1945

[4] Bloch, K., and Rittenberg, D., *J. Biol. Chem.*, **159**, 45 (1945).
[5] Fischer, H., and Fink, E., *Z. physiol. Chem.*, **280**, 123 (1944).

COLORIMETRIC DETERMINATION OF VITAMIN A

Sirs:

In view of the recent note[1] which came from the press June 5, 1945, we wish to report, in advance of a more detailed article, our experience in the past 3 months with a new method independently discovered for determining vitamin A.

Both glycerol α,γ-dichlorohydrin and mixed glycerol dichlorohydrin (approximately 70 per cent α,β and the rest α,γ)[2] will react with vitamin A in either of the solvents ethylene dichloride or chloroform but will not react well in ethyl alcohol or isopropyl alcohol. The reaction with vitamin A follows Beer's law up to at least 250 U. S. P. units per ml. (520 or 660 mμ filters) under our conditions. Vitamin A has been determined in hexa multivitamin tablets containing vitamins A, B_1, B_2, C, and D, and niacinamide with no interference from these vitamins. Vitamin D does not interfere with the vitamin A determination with a ratio of A to D of 5 : 3 (in terms of U. S. P. units) but does interfere when the ratio is 1 : 2.5. Vitamin A in fish liver oils and multivitamin tablets determined by our procedure shows agreement within 5 to 10 per cent with the vitamin A calculated by using the extinction coefficient at 328 mμ ($E^{1\%}_{1\,\text{cm.}} \times 2000$).

For the determination 2 drops of concentrated HCl followed by 5 ml. of the dichlorohydrin are added to 10 ml. of a chloroform solution of vitamin A containing 600 to 3000 U. S. P. units. The vitamin A solution is made by a simple dilution of vitamin A oil with chloroform or by filtering a chloroform extract of ground multivitamin tablets. The mixture is shaken for 5 minutes. The color, which is first blue, passes through blue, blue-green, pink, rose, violet, and dirty blue, and in 30 minutes appears as a clear blue-green which can be read in the photoelectric colorimeter (660 mμ filter). By reference to a standard curve based on spectrophotometric assays the vitamin A content is easily determined. The blue-green color so developed with one lot of dichlorohydrin has been stable in the light for almost 24 hours, while other lots give a color stable for approximately 3 to 4 hours. If a standard run at the same time (on one of the latter lots of reagent) is allowed to fade along with the unknown sample, the compared readings 18 hours later still give vitamin A values almost identical with those calculated from the readings taken at 30 minutes.

With the Beckman spectrophotometer and 20 mμ intervals the reaction with vitamin A plus the HCl shows four maximum density peaks between

[1] Sobel, A. E., and Werbin, H., *Federation Proc.*, **4**, 104 (1945).

[2] As a word of caution, the dichlorohydrins are reputed to be strong heart and respiratory depressants.

400 and 800 mμ within 10 to 20 minutes after the start of the reaction. These peaks are approximately at 440, 580, 640, and 740 mμ with a deep minimum density at 500 mμ. When the reaction is allowed to take place without HCl, the curve shows a maximum density peak at 560 mμ and then after a low density at 620 mμ a continuous rise in density to 800 mμ. With the same quantity of vitamin A, the addition of the acid makes the density readings of the four maximum peaks over 2 to 3 times the density reading of the single maximum peak obtained without the addition of acid.

Office of Marketing Services
War Food Administration
Washington

LOUIS FEINSTEIN

Received for publication, June 18, 1945

THE NITROGENOUS CONSTITUENTS OF FLAXSEED

II. THE ISOLATION OF A PURIFIED PROTEIN FRACTION*

By BRUNO VASSEL AND L. L. NESBITT

(From the Department of Agricultural Chemistry, North Dakota Agricultural College and Experiment Station, Fargo)

(Received for publication, April 9, 1945)

Data are available on the purified proteins of many of the common seeds of commercial importance. It is therefore surprising that little is known about the proteins of flaxseed, particularly because flax is an economically important crop in many sections of the world. Linseed meal, the nearly oil-free residue of the ground flax seeds, is rich in nitrogen and should command a more important position in the economy of the flax industry than it has up to now.

The earliest report on flaxseed proteins is furnished by Osborne (1) who extracted lipid-free linseed meal with either water, 10 per cent NaCl solutions, or dilute alkali. He recorded that the addition of very dilute acetic acid to aqueous extracts precipitated some proteins which were soluble in an excess of the acid but which were again precipitated upon the addition of salt. Acidification of saline extracts with acetic acid precipitated proteins, insoluble in an excess of the acid, but partially soluble in an excess of cold HCl, and completely soluble in hot HCl. No further attempt to fractionate the flaxseed proteins has come to our attention since Osborne's observations were published in 1892, but the literature contains a number of peptization studies with various aqueous solvents (2–4)[1] and several partial amino acid analyses of the bulk of the crude proteins (5–8).

This report presents a description of the isolation, purification, and of some of the chemical and physical properties of one of the flaxseed proteins, which, according to data now available, appears to be the major protein of linseed meal. We propose naming this protein linin, derived from the botanical name of flax, *Linum usitatissimum*. Throughout this investigation our primary aim has been to obtain linin as a homogeneous entity; consequently, less attention was paid to yield than to purity. We consider the procedure finally adopted as a reasonably satisfactory one, subject, however, to further improvements. We shall also place on record some of the early difficulties which we encountered with water or dilute salt extracts

* Published by permission of the Director, North Dakota Agricultural Experiment Station. This work was carried out under Purnell Project 95, "The chemistry of flaxseed."

[1] Painter, E. P., and Nesbitt, L. L., unpublished.

of linseed meal. These observations may, perhaps, be useful to others who wish to investigate further protein extraction methods with aqueous solvents. We have particularly in mind those in the flaxseed industry who have long hoped to make better use of linseed meal.

Flaxseed contains, in addition to linin, several other proteins. One of these in particular is obtainable in fair amounts. We shall occasionally mention this protein during the discussion and shall refer to it as conlinin. A detailed description of its isolation and purification will be submitted later.

EXPERIMENTAL

The seeds used were a mixture of samples received from agronomists who were working to improve flax varieties. They consisted of the more common flax varieties generally grown in the north central states. The seeds, cleaned from gross impurities by sieving through a 20 mesh screen, were ground in a laboratory model roller mill. The ground meal contained 5.7 to 5.8 per cent moisture, 3.9 to 4.0 per cent ash, 4.9 to 5.0 per cent nitrogen (ash- and moisture-free[2]), and 37 per cent lipids, extracted in a Soxhlet apparatus for 24 hours with petroleum ether (Skellysolve, 40-60). The nitrogen content of the fat-free meal was 6.80 to 6.85 per cent, which by calculation indicates no loss in nitrogenous constituents during lipid extraction.

Water and Aqueous Salt Extractions—The meal swells upon the addition of water or 5 per cent salt solutions but can readily be stirred or shaken at a ratio of 10 gm. or less of meal to 100 cc. of solvent. Aqueous solutions of the meal have pH values below neutrality, which slowly decrease further to an occasional low near pH 4.8. Salt solutions, either 5 per cent NaCl, 5 per cent Na_2SO_4, or 7 per cent $(NH_4)_2SO_4$, have the same approximate initial pH value but the tendency of the pH to decrease further is not as pronounced as in the absence of salts. For 100 gm. of meal, from 2.5 to 3.5 cc. of 5 N NaOH are needed to maintain a pH of 7.0. In all of the extractions here reported, 10 gm. of meal were shaken first with 100 cc. and then with 50 cc. portions of the solvents for 1 hour periods. Longer shaking increases but little the amount of extracted nitrogen. When water and 5 per cent Na_2SO_4 solutions are compared, the extractable nitrogen is more closely a function of pH than of the solvent used. Table I presents some data indicative of the influence of pH on the amount of nitrogen extracted in water and 5 per cent Na_2SO_4 at pH values of 5.5, 7.0, 8.2 (Na_2CO_3), and 10.2 (NaOH).

The supernatant of an extract below pH 7 is cloudy and somewhat yellow; alkaline extracts are deep yellow to orange in color. The crude pro-

[2] All analytical values and yields, unless otherwise stated, have been corrected for ash and moisture content.

teins which precipitate from the extracts at pH 3.9 to 4.1, and which centrifuge out readily, are white in appearance and give no indication that they contain material which upon re-solution in dilute NaOH gives rise to very slimy solutions of a yellow to red-brown color. Proteins obtained from water or 5 per cent salt extracts at pH values below neutrality carry, upon solution at pH 10.2, considerably less of the colored and slimy impurities than when alkaline solutions are used in the original extraction. This is clearly reflected in the nitrogen values of some of the impure preparations of proteins isolated from the original extracts, as shown in the first four lines of Table VI. Filtration of the extracts raises the nitrogen content of the isolated protein, but a large part of the protein material is filtered out with

Table I

Nitrogen Peptized from 10 Gm. of Fat-Free Linseed Meal by Two Extractions of 100 and 50 Cc. of Water or 5 Per Cent Na_2SO_4 at Different pH Values

The calculations are based on the actual volume of supernatant recovered after centrifugation.

Solvent used	Solution maintained at pH	N found in		Total N extracted
		1st extract	2nd extract	
		per cent	*per cent*	*per cent*
H_2O	5.5	43.4	9.5	52.9
"	7.0	54.2	11.0	65.2
"	8.2	59.3	18.9	78.2
"	10.2	68.7	18.7	87.4
Na_2SO_4	7.0	53.8	11.5	65.3
"	8.2	55.4	17.6	73.0
"	10.2	65.3	21.9	87.2

the slimy impurities. Highest protein yields of filtered salt extracts were obtained when 2 gm. of Hyflo Filter-Cel (Johns-Manville) were stirred into 1000 cc. of solution at pH 10.2, and filtered by suction through a mat of approximately 2 mm. thickness of Filter-Cel supported on gauze. While this method is better than filtration through filter paper, the yield is still very poor, and the frequent reextractions are too inefficient to make this method acceptable. Osborne (1) recognized these difficulties in his statement (p. 655), "These operations [purification of 5 per cent NaCl extracts] were in all cases greatly prolonged on account of the gum contained in the seed, which rendered filtration extremely difficult and slow."

To avoid misunderstanding, we wish to state, however, that the removal of the slimy impurities is necessary only when it is desired to isolate the individual flaxseed protein fractions as homogeneous products, and in the highest attainable state of purity. Actually, from a quantitative stand-

point, the slimy impurities make up only a small part of the weight of the bulk of the crude proteins which precipitate when the pH of the original salt extracts is brought to 4.5 with 5 N HCl.

Inasmuch as the residual, unextracted nitrogen of the meal is appreciable, ethanol extractions were made on the residue after 76.1 per cent of the original nitrogen has been extracted with 5 per cent NaCl. As shown in Table II, some of the residual nitrogen is alcohol-soluble. When aliquots of these extracts were diluted with 5 volumes of water, acidified, and trichloroacetic acid was added to a 10 per cent final concentration, opalescence occurred, but no precipitate was formed during the next 24 hours. This was interpreted as indicating the absence of alcohol-soluble proteins in linseed meal after extraction with salt solutions.

Table II

Ethanol Extraction of Linseed Meal Previously Extracted Four Times at pH 6.9 with 5 Per Cent NaCl

A total of 76.1 per cent of the original nitrogen had been extracted. The residual nitrogen content of the meal was 1 63 per cent.

Ethanol concentration in aqueous solvent, %	42	50	58	70	75	80
N extracted per 100 gm meal, *gm.*	0.146	0.139	0 118	0.112	0.096	0.080
Calculated N extracted, %	8.95	8 53	7.24	6.87	5.88	4.90

Extraction with Organic Solvents. Ethylene Glycol—After it became evident that it was very difficult to remove the slimy impurities from proteins extracted by water or salt solutions, our attention turned to organic solvents in the hope of finding one which would either precipitate the flaxseed proteins, or the gum, without precipitating both together. Preliminary tests with a number of organic solvents indicated that the gum consisted of at least two components, either of which is responsible for the slimy consistency of alkaline, aqueous extracts. One of these gummy substances could be precipitated by the addition of ethylene glycol to aqueous or salt extracts of linseed meal. The precipitate was flocculent but settled unsatisfactorily. It required 30 minutes or longer centrifugation at fairly high speeds to pack. The precipitate, in a representative trial run, contained 5.68 per cent of the nitrogen of the extract. Since centrifugation was unsatisfactory, the minimum ratio of 0.2 M phosphate buffer at pH 7.2 to ethylene glycol was investigated which would extract a maximum amount of nitrogen without also extracting the gummy impurity. The absence of the latter was ascertained by adding enough glycol to the extract to give a final concentration of 2 volumes to each volume of buffer. These data are shown in Table III.

It is evident that beginning with a ratio of 1:1.25 of buffer to ethylene

glycol none of the ethylene glycol-insoluble material is extracted. Furthermore, the amount of nitrogen in solution is constant for the range tested and amounts to about 65 per cent of the total nitrogen in three extractions. This is approximately equal to the nitrogen obtained by two extractions

Table III

Nitrogen Peptized from 10 Gm. of Meal with 100 and 50 Cc. Portions of Phosphate Buffer-Ethylene Glycol at pH 7.2

The final phosphate concentration was about 0.08 M.

Ratio of phosphate to ethylene glycol	Total N extracted in				Pptn. of gum with 2 volumes ethylene glycol
	1st extraction	2nd extraction	3rd extraction	Total	
	per cent	*per cent*	*per cent*	*per cent*	
1:0.50	44.4				Visible ppt.
1:1.00	39.1				" "
1:1.10	41.2	20.7			" "
1:1.18	40.7	19.4			Doubtful ppt.
1:1.25	40.2	17.6	7.7	65.5	No ppt.
1:1.40	40.7	18.3	6.3	65.3	" "
1:1.50	39.4	18.3	6.4	64.1	" "

Table IV

Nitrogen Peptized from 10 Gm. of Meal with 100 and 50 Cc. Portions of 1:1.4 Phosphate Buffer-Ethylene Glycol at Various pH Values

The final phosphate concentration was about 0.08 M.

pH of buffer-glycol mixture	pH of extract after		Total N extracted in		
	1st extraction	2nd extraction	1st extraction	2nd extraction	Total
			per cent	*per cent*	*per cent*
Water	5.6	5.3	32.8	10.4	43.2
6.5	6.3	6.6	31.2	12.0	43.2
6.8	6.7	6.9	31.2	12.3	43.5
7.7	7.2	7.7	40.4	12.4	52.8
8.2	7.7	8.0	38.7	13.4	52.1
8.6	7.8	8.0	40.2	12.3	52.5
9.0	7.8	8.1	38.8	13.6	52.4
9.5	7.9	8.2	39.0	12.4	51.4
10.1	8.0	8.8	38.8	12.4	51.2
10.5	9.6	10.0	40.2	12.4	52.6
10.8	10.3	10.4	40.7	12.5	53.2

with water or 5 per cent Na_2SO_4 at pH 7. Increasing the pH of the buffer above 7 has little influence on the amount of extractable nitrogen, as Table IV shows.

Not all of the nitrogen extracted by ethylene glycol is protein nitrogen.

A glycol extract, after dilution with 4 volumes of water, containing 0.340 gm. of nitrogen per 100 cc., and representing 60.2 per cent of the original 6.39 per cent nitrogen of the meal, was fractionated with 20 per cent trichloroacetic acid, with 20 per cent sulfosalicylic acid, and by dialysis followed by trichloroacetic acid precipitation. The data are shown in Table V. It is evident that trichloroacetic acid and sulfosalicylic acid precipitate about the same amounts of nitrogen, leaving 21.4 and 22.1 per cent of non-protein nitrogen, respectively, in solution. If the extract is first dialyzed and then precipitated with trichloroacetic acid, 28 per cent of non-protein nitrogen is found. We presume that the higher value after dialysis represents some decomposition of the proteins by microorganisms. Assuming that 21.7 per cent is a close approximation of the non-protein nitrogen in the extract, then 78.3 per cent of the extracted nitrogen (or 47.1 per cent of the original nitrogen of the meal) is protein nitrogen. Exactly how much of this protein nitrogen belongs to linin we do not know. Attempts to fractionate linin from conlinin by a single separation into soluble (conlinin) and insoluble (linin) fractions at pH 5.7 gave erratic results which we can only explain by our later observation that both proteins have a strong tendency to coprecipitate at their respective isoelectric points.

Table V

Distribution of Nitrogen in Twice Extracted Linseed Meal, with Ethylene Glycol-Phosphate Buffer As Solvent

	N calculated from	
	N actually extracted (60.2 per cent of total N)	Total N in meal (6.39 per cent)
	per cent	*per cent*
N not extracted by 2 extractions		39.8
Extracted N not precipitable by trichloroacetic acid	22.1	
" " " " " sulfosalicylic acid	21.4	
Non-protein N, average of sulfosalicylic and trichloroacetic acid methods	21.7	13.1
Protein N in extract, average (by difference)	78.3	47.1
Total	....	100.00
N dialyzable in 6 days	13.8	8.3
" not dialyzable and not pptd. with trichloroacetic acid	14.2	8.6
Protein N after dialysis and trichloroacetic acid treatment	72.0	43.3

Ethylene glycol extracts are deep yellow-red in color. When, after dilution with 4 volumes of water, the bulk of the proteins is precipitated at pH

4.5, part of the pigment-producing substances becomes colorless and precipitates with the proteins, the other colored part remaining in solution. Upon solution of the proteins at values above pH 9, the pigments regain their former color, giving the entire solution a muddy brown appearance. If now filtered, all of the slimy impurity but only part of the pigments is retained by the filter paper. Filtration is considerably more rapid but an appreciable part of the proteins is again lost, although not as much as when the glycol-insoluble impurity was also present. Accordingly, we searched for a further precipitating agent for the second gum-like substance.

Dioxane—The addition of 100 cc. of dioxane to 100 cc. of glycol extract, diluted with 100 cc. of water, and maintained at pH 10.1 to 10.2, precipitates the gum nearly quantitatively. The gum flocculates out and can be removed by centrifugation for 40 minutes. When large volumes are used, it is more practical to allow the impurity to settle during 24 hours, to siphon off and filter the supernatant, and to centrifuge only the residue. In a trial run, 0.8 gm. of a very slimy substance was isolated from a glycol extract of 100 gm. of meal, containing 9.44 per cent (uncorrected) nitrogen. From it 0.4 gm. of proteins was isolated. A second sample of this gummy material, after more extensive purification, contained 2.35 per cent nitrogen (uncorrected), and 23.95 per cent ash.

TABLE VI

Nitrogen Content of Flax Proteins Obtained by Various Extraction Procedures

All values are corrected for moisture and ash.

Type of solvent used for extraction	Method of partial purification	Protein isolated per 100 gm. meal	N in protein
		gm.	*per cent*
H_2O at pH 10.2	2 reprecipitations	16.2	12.69
" " " 5.5	2 "	7.1	16.28
5% Na_2SO_4 at pH 5.5	2 "	7.4	16.28
5% " " " 5.5	Filtered, then pptd. once	1.7	16.60
1:1.4 PO_4 buffer-glycol	Filtration, 4 pptns.	5.6	16.79
Same	Dioxane treatment, 4 filtrations and pptns.	5.2	17.02

The supernatant, after removal of the gum, shows slight opalescence, and is strongly honey-colored. After dilution with 1.5 volumes of water and acidification to pH 4.5 to 4.6, linin and conlinin precipitate together as a white material, leaving a perfectly clear, honey-colored supernatant. Since small amounts of the dioxane-insoluble material are precipitated with the proteins, a second dioxane treatment, described in detail later, is necessary.

In a trial run, 5.2 gm. of linin and conlinin were isolated from a dioxane-treated glycol extract of 100 gm. of meal, after four reprecipitations at pH 4.5 from filtered water solutions of pH 10.2. These proteins contained 17.02 per cent nitrogen. Table VI summarizes the yields and the per cent nitrogen content of the preparations isolated by the previously discussed extraction procedures. It is evident that the purity of the product improved with the adoption of each new procedure.

The use of dioxane has still another advantage. The honey-colored ethylene glycol extracts carry, among others, a red pigment which, upon

TABLE VII

Nitrogen Peptized by Two Extractions of 10 Gm. of Linseed Meal (100 and 50 Cc.) by Formic Acid-Water Mixtures

The per cent nitrogen extracted is calculated from the volumes of supernatant actually recovered after centrifugation.

Formic acid used	N extracted		
	1st extraction	2nd extraction	Total
per cent	*per cent*	*per cent*	*per cent*
87.0*	62.7	18.5	81.2
78.3	60.7	18.5	79.2
69.6	59.0	17.3	76.3
60.9	55.9	15.3	71.2
52.2	52.7	15.4	68.1
43.5	48.3	14.4	62.7
34.8	42.4	13.3	55.7
26.1	36 3	12.0	48.3

* 87.0 per cent according to the manufacturer's label. All other formic acid percentages are calculated accordingly.

precipitation of the proteins on the acid side in the absence of dioxane, is to some extent carried down but is not in the presence of dioxane.

Formic Acid—During a search for organic solvents which would extract the proteins from linseed meal without extracting carbohydrate impurities at the same time, the paper of Albanese *et al.* (9) on the use of formic acid as a peptizing agent of vegetable proteins came to our attention. The concentrated acid (87 per cent) extracted about the same amounts of nitrogen from linseed meal as did aqueous or salt solutions at pH 10.2. After even a slight dilution, its peptizing powers decreased rapidly; illustrative data are shown in Table VII.

Unfortunately, sufficient gum was extracted to discourage the use of formic acid. One incidental observation, however, may prove of considerable value. Purified linin dissolves very rapidly in 87 per cent formic acid

without alteration of its solubility, as far as we have been able to determine. The solubility of conlinin, on the other hand, becomes altered to the extent that only a milky appearing solution can be recovered after careful neutralization to pH 4.5 at temperatures below 10°. This suggested a means of increasing the efficiency of the final purification procedure for linin, as discussed in the next section.

Recommended Procedure for Isolation of Linin—250 gm. of petroleum ether-extracted linseed meal are mechanically shaken or stirred for two 1 hour periods with 2500 and 1500 cc. of a buffer-glycol mixture, prepared from 417 cc. of 0.2 M Na_2HPO_4-NaH_2PO_4 at pH 7.2 to which are added 583 cc. of ethylene glycol. Centrifugation after each shaking period yields a total of 3000 to 3100 cc. This solution has been kept for several months at room temperature, without noticeable growth of microorganisms. Upon standing, the pH slowly drops and some of the proteins will precipitate. This is of no consequence, since the proteins will readily redissolve in the next step of the procedure.

The glycol extract is now brought to pH 10.0 to 10.2 by the dropwise addition of 25 per cent NaOH, with constant mechanical stirring of the solution. To each 1000 cc. of alkaline extract are added 1000 cc. of distilled water, and slowly 1000 cc. of dioxane while the solution is stirred and maintained at pH 10.2. The gum becomes increasingly insoluble with increasing alkalinity, but to go above pH 10.2 is of no advantage. After about 2 hours, flocculation begins and is complete in about 24 hours. The supernatant, which is slightly opalescent and strongly honey-colored, is siphoned off and filtered. The residue is centrifuged at high speeds for 40 minutes and the supernatant added to the filtrate. After dilution with 1.5 volumes of distilled water and acidification to pH 4.5 to 4.6 with 5 N HCl, the white proteins (linin plus conlinin) settle out readily in a few hours, leaving a clear light yellow supernatant from which the small albumin and globulin fractions can be salted-out. This will be described in a subsequent report. The precipitated proteins are dissolved in 2000 cc. of distilled water at pH 10.2 (NaOH). For every 1000 cc. of solution 380 cc. of dioxane are added, the pH being maintained at 10.1 to 10.2. The small amounts of gummy material which escaped the first dioxane treatment are filtered off with Schleicher and Schüll No. 725 filter paper, the best paper for flax protein work that we have found. The filtrate is brought to pH 4.5, the precipitated proteins centrifuged, and the now only faintly yellow supernatant is discarded. The proteins are redissolved in water at pH 10.0 to 10.2, reprecipitated first at pH 5.7 (linin) and then at pH 4.5 (conlinin). The latter is redissolved once more at pH 10.2, reprecipitated at pH 5.7, and the proteins obtained at this pH added to the first batch. It is possible to obtain linin almost free from other proteins by repeated solution at pH 10.2

and reprecipitation at pH 5.7. The last traces of protein impurities are, however, difficult to remove, unless the rather severe formic acid treatment is used.

Unless later proved otherwise, we now believe that it is safe to dissolve linin (from 250 gm. of meal) in about 800 cc. of 87 per cent HCOOH, to stir it at room temperature for 15 to 20 hours, and to recover the now conlinin-free linin by dilution with an equal volume of water, the temperature being maintained below 10° with additions of ice directly while 5 N NaOH is added dropwise with mechanical stirring until the pH is above 4. A final adjustment to pH 5.7 is made after the solution has come to room temperature. In order to wash out adhering decomposition products of the previous protein impurities, the linin is once more dissolved in 1000 cc. of water at pH 10.2 and reprecipitated at pH 5.7. The precipitate can be dried without a noticeable change in its solubility properties by washing it with ethanol and ether.

Data presented in Table VIII were obtained from two linin preparations, neither of which had been treated with dioxane. The use of dioxane in the removal of the second of the gummy impurities is a rather recent development, and while we now have dioxane-treated protein preparations of nitrogen content equal to our purest preparations by other methods, no solubility data have as yet been obtained on these as criteria of purity. The proteins discussed in Table VIII were, in one case, prepared from a six times filtered and reprecipitated ethylene glycol extract, treated with 87 per cent HCOOH, as previously discussed. The final product was dried with alcohol and ether. The other linin preparation of Table VIII was isolated from an ethylene glycol extract. A total of eighteen precipitations at pH 5.7, and peptizations at pH 10.2, followed by filtration each time, was performed. After being dialyzed and dried from the frozen state in a high vacuum (10), this preparation contained 17.15 per cent nitrogen (micro-Kjeldahl (11)), 0.64 per cent sulfur (alkaline permanganate ashing (12)), 1.26 per cent phosphorus (Berggren's (13) modification of Fiske and Subbarow's (14) method), and 0.51 per cent carbohydrates (Hewitt's (15) adaptation of Sørensen and Haugaard's (16) method, with an equimolar mixture of galactose and mannose as the arbitrary reference solution). All values are calculated on an ash- (1.49 per cent) and moisture- (2.90 per cent) free basis. When tested for homogeneity by the application of Gibb's phase rule to solubility data in accordance with Northrop[3] and Kunitz's (17) method, as modified by Butler (18), the preparation was found to be contaminated by small amounts of one or more other proteins. The entire preparation was therefore shaken in M/15 phosphate

[3] We wish to express our gratitude to Dr. J. H. Northrop for valuable advice and suggestions with respect to the handling and interpretation of the solubility data.

buffer at pH 5.7, containing 0.25 saturated Na_2SO_4, in a constant temperature bath at 25°. The Na_2SO_4 served as a bacteriostatic agent. After each 48 hour shaking period the solution was centrifuged and the supernatant

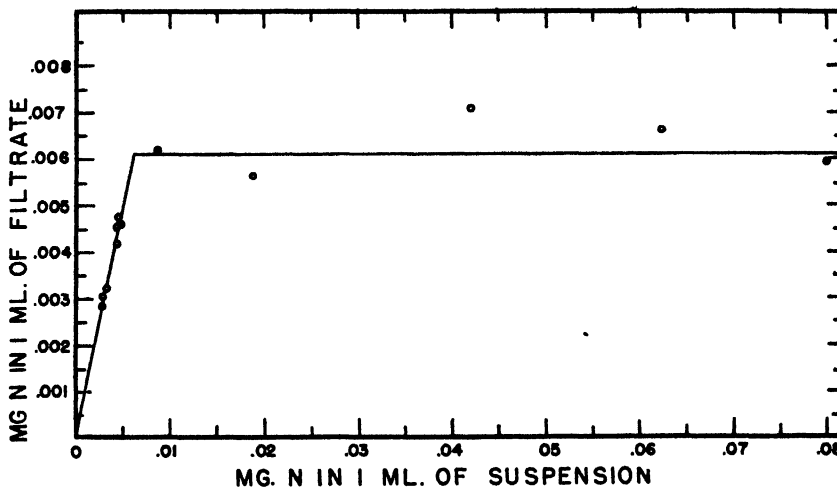


FIG. 1. Solubility of linin in M/15 phosphate-0.25 saturated sodium sulfate at pH 5.7 and 25° in the presence of increasing quantities of solid phase.

TABLE VIII

Chemical and Physical Data on Two Linin Preparations

All values are calculated on an ash- and moisture-free basis.

	Linin prepared by	
	Equilibrating in PO_4 buffer	HCOOH treatment
Nitrogen, %	16.99	17.01
Sulfur, %	0.61	0.59
Phosphorus, %	<0.01	<0.01
Carbohydrate, %	0.54	*
Isoelectric point (from Fig. 2)	5.75	
Approximate solubility in distilled water adjusted to indicated pH with HCl or NaOH	Above 6.9 Below 3.6	

* Hewitt's method calls for the addition of 60 per cent H_2SO_4 to the protein. When this is done, a deep violet color develops in the HCOOH-treated protein, even in the absence of orcinol, which makes a colorimetric carbohydrate determination impossible.

analyzed for nitrogen. After the eighth shaking period, constant nitrogen values were obtained. The product was now tested for purity by solubility measurements and was found to be homogeneous (Fig. 1) from the slope of

1, and from the constant solubility when fully saturated. Chemical and physical data on the dialyzed (16 days) preparation after it was dried from the frozen state are given in Table VIII, which also contains the corresponding values for the preparation treated with formic acid. An interesting feature of these data is the absence of phosphorus in both of the purified preparations. It should be recalled that one of these preparations, prior

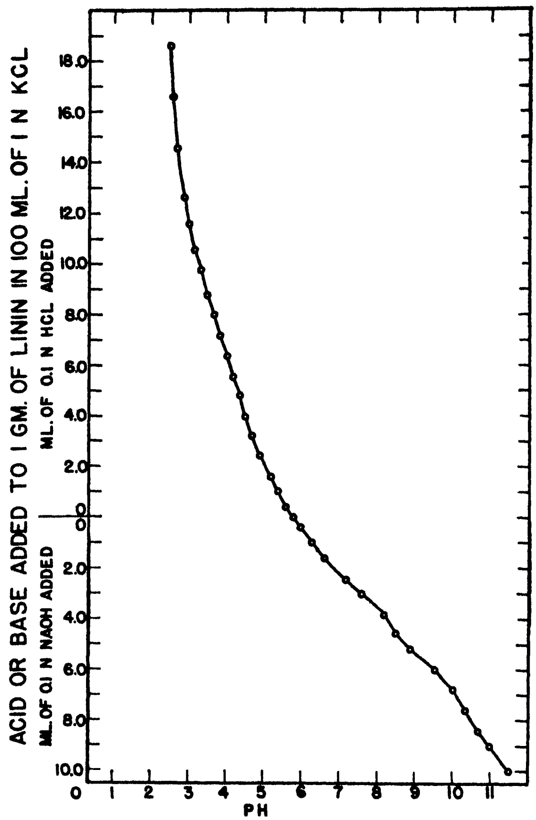


FIG. 2. Electrometric titration curve of linin between pH 2.5 and 11.5

to equilibration to constant solubility, contained 1.26 per cent phosphorus. It is unlikely that this was due to contamination by inorganic phosphorus for the following reasons. Eighteen reprecipitations at pH 5.7, followed by a 16 day dialysis, should have removed most of the inorganic phosphorus; the nitrogen content of the protein prior to the removal of the phosphorus was as high as that of the final homogeneous product; lastly, the two crude protein preparations isolated from a 5 per cent Na_2SO_4 extract

at pH 5.5 and from dilute alkali at pH 10.2, shown in Table VI, contained 0.45 and 0.54 per cent phosphorus, respectively, but neither of these had been extracted with phosphate-buffered solutions. We are inclined to believe that a phosphorus- and nitrogen-rich substance was removed by the equilibrating procedure, and by the HCOOH treatment.

In order to obtain a better approximation of the isoelectric point an electrometric titration of linin in 1 N KCl was performed between pH 2.5 and 11.5 at 25°, shown in Fig. 2. A Coleman glass electrode assembly was used. No further glass correction was applied for the electrode in alkaline solutions. According to the titration data the isoelectric point lies at or close to pH 5.75, which confirms our previous observation that the maximum insolubility of linin appears to be near pH 5.7.

DISCUSSION

It is difficult to compare our data with those of Osborne (1). It appears that the protein which Osborne describes as insoluble in dilute and strong acetic acid, but partially soluble in strong HCl, resembles most closely the properties of our linin preparations. All of Osborne's "globulins" crystallized readily during dialysis as octahedra or spheroids; none of our linin or conlinin preparations are crystalline. The least understandable differences between Osborne's and our preparations are the analytical values for nitrogen and to a lesser extent sulfur. His globulins, calculated on an ash-free basis, contained 18.34 to 18.95 per cent nitrogen and 0.73 to 0.99 per cent sulfur. These figures are appreciably higher than ours and we are at a loss to account for these differences.

SUMMARY

The isolation of a protein from flaxseed is described. It appears to be the major protein component. The purified protein, named linin, according to solubility data, is a homogeneous product. It has an isoelectric point at or near pH 5.75, and contains 17.0 per cent nitrogen, 0.6 per cent sulfur, and 0.54 per cent carbohydrate.

BIBLIOGRAPHY

1. Osborne, T. B., *Am. Chem. J.*, **14**, 629 (1892).
2. Staker, E. N., and Gortner, R. A., *J. Phys. Chem.*, **35**, 1565 (1931).
3. O'Hara, L. P., and Saunders, F., *J. Am. Chem. Soc.*, **59**, 352 (1937).
4. Smith, A. K., Circle, S. J., and Brother, G. H., *J. Am. Chem. Soc.*, **60**, 1316 (1938).
5. Foreman, F. W., *J. Agr. Sc.*, **3**, 358 (1910).
6. Hamilton, T. S., Uyei, N., Baker, J. B., and Grindley, H. S., *J. Am. Chem. Soc.*, **45**, 815 (1923).
7. Jones, D. B., Gersdorff, C. E. F., and Moeller, O., *J. Biol. Chem.*, **62**, 183 (1924–25).

8. Kratzer, F. H., *J. Biol. Chem.*, **156**, 507 (1944).
9. Albanese, A. A., Wagner, D. L., Frankston, J. E., and Irby, V., *Ind. and Eng. Chem., Anal. Ed.*, **16**, 609 (1944).
10. Hays, E. E., and Koch, F. C., *Science*, **95**, 633 (1942).
11. Ma, T. S., and Zuazaga, G., *Ind. and Eng. Chem., Anal. Ed.*, **14**, 280 (1942).
12. Pollock, R. N., and Partansky, A. M., *Ind. and Eng. Chem., Anal. Ed.*, **6**, 330 (1934).
13. Berggren, R. E. L., *J. Biol. Chem.*, **95**, 461 (1932).
14. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
15. Hewitt, L. F., *Biochem. J.*, **31**, 360 (1937).
16. Sørensen, M., and Haugaard, G., *Biochem. Z.*, **260**, 247 (1933); *Compt.-rend. trav. Lab. Carlsberg*, **19**, 1 (1933).
17. Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, **13**, 781 (1930). Kunitz, M., and Northrop J. H., in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, **6**, 325 (1938).
18. Butler, J. A. V., *J. Gen. Physiol.*, **24**, 189 (1940).

ON THE REVERSIBILITY OF LEVULAN SYNTHESIS BY BACILLUS SUBTILIS

By MICHAEL DOUDOROFF and ROLIE O'NEAL

(*From the Department of Bacteriology, University of California, Berkeley*)

(Received for publication, April 25, 1945)

Since the discovery of the phosphorolytic breakdown and synthesis of starch, glycogen, and sucrose, the attention of many cell physiologists has been centered on the rôle of phosphoric esters of sugars as precursors of polysaccharides and of disaccharides in plant and animal metabolism. While the importance of phosphoric esters in metabolic processes is well established, it must be realized that synthetic mechanisms not directly involving such compounds may be wide-spread in nature. For this reason, the bacterial syntheses of levulan and of dextran from sucrose are of special interest, since the formation of these polysaccharides is easily effected with cell-free enzyme preparations and does not appear to involve the accumulation of phosphoric esters.

Hehre (1) has obtained a crude enzyme preparation from cultures of *Leuconostoc mesenteroides* which catalyzes the reaction

$$\underset{\text{Sucrose}}{nC_{12}H_{22}O_{11}} \rightarrow \underset{\text{Fructose}}{nC_6H_{12}O_6} + \underset{\text{Dextran}}{(C_6H_{10}O_5)_n}$$

The same species has been shown by Kagan, Latker, and Zfasman (2) to cause the phosphorolysis of sucrose with the formation of fructose and glucose-1-phosphate, a reaction which has been studied with another bacterium and used as the basis for synthesis of sucrose and related disaccharides (3–6). It therefore seemed reasonable to suppose that dextran would arise as a condensation product of glucose-1-phosphate. Hehre (7) has shown that this is not the case, since his enzyme was inactive toward the phosphoric ester.

Hestrin and Avinieri-Shapiro (8) have shown a similar situation in the synthesis of levulan from sucrose by *Aerobacter levanicum*. The crude enzyme preparation obtained by them catalyzes the reaction

$$\underset{\text{Sucrose}}{nC_{12}H_{22}O_{11}} \rightarrow \underset{\text{Levulan}}{(C_6H_{10}O_5)_n} + \underset{\text{Glucose}}{nC_6H_{12}O_6}$$

In addition, some inversion of sucrose occurs; so that the authors were prompted to write a more complicated but hardly a more useful expression of the observed facts, in which both the synthetic and hydrolytic cleavage products are included. They found the ratio of sucrose hydrolyzed to su-

crose used in levulan synthesis to approximate unity. Their dialyzed enzyme was found to be active in the absence of added phosphate, indicating that no accumulation of phosphoric esters is necessary for the synthesis of levulan. Hestrin and Avinieri-Shapiro's studies of the properties of their enzymes were as thoroughly carried out as the crude material permits and leave little to be desired. One question, however, was left unanswered by these authors; namely, whether the reaction is reversible. They could not show that the reverse reaction occurred when glucose and levulan were added to the enzyme. The question is a rather important one, since thermodynamic considerations would lead one to expect an equilibrium in which demonstrable quantities of the reactants would occur, and an irreversible reaction would be incompatible with our knowledge of enzyme chemistry. For this reason, it seemed of interest to reinvestigate the problem to see whether reversibility could be demonstrated for the process.

EXPERIMENTAL

Instead of *Aerobacter* cells, the source of enzyme used in the present studies was the culture medium in which *Bacillus subtilis*[1] had been grown in the presence of sucrose. The ability of many *Bacilli* to form levulan and the occurrence of the active principle in the cell-free medium have been familiar to every bacteriologist since the pioneering work of Beijerinck (9).

The properties of the enzyme preparations thus obtained are very similar to those described by Hestrin and Avinieri-Shapiro. Both synthesis of levulan and hydrolytic cleavage of sucrose appear to go on simultaneously, but the ratio between the products of hydrolysis and synthesis was found to vary considerably, depending on the environmental conditions and the method of treatment of the preparations. The optimum is around pH 5, the synthesis of levulan proceeds readily without added phosphate, and the rate of synthesis is reduced by the addition of glucose. Unlike their preparations, however, the *Bacillus subtilis* culture medium was found to effect a slow hydrolysis of levulan, indicating that the accumulated polysaccharide might be used as an extracellular reserve product by the organism. As in Hestrin's studies, the reversibility of levulan synthesis could not be demonstrated directly with these preparations. It seemed likely that a major difficulty was the high molecular weight and hence the relatively low molar concentration of the polysaccharide in solutions which can be readily handled. It seemed desirable, therefore, to displace the equilibrium to the left by removing any traces of sucrose which might be

[1] The culture used in these studies was found in the departmental collection and kindly identified for us by Dr. Nathan R. Smith of the Bureau of Plant Industry of the United States Department of Agriculture as typical *Bacillus subtilis*.

formed by the reverse reaction. This was done by the addition of yeast invertase, in the presence of which indirect evidence was obtained for the occurrence of a back reaction and for a new mechanism of sucrose synthesis.

Experiments with Concentrated Culture Medium—Cultures of *Bacillus subtilis* grown in a medium containing sucrose were used as a source of both levulan and enzyme for the experiments. The medium was prepared with 5 per cent sucrose, 0.05 per cent $MgSO_4 \cdot 7H_2O$, 5 per cent yeast autolysate (by volume), and M/30 KH_2PO_4-Na_2HPO_4 (Sörensen) buffer at pH 6.8. The organisms were allowed to grow for 20 hours at 30° with constant agitation on a rotary shaker, and removed by centrifugation. The supernatant was then dialyzed for 15 hours against running tap water, buffered with 0.002 M citrate buffer at pH 5.5, and concentrated by vacuum distillation at 10° to about one-tenth the original volume. The concentrate was again centrifuged and the supernatant dialyzed against running distilled water on a rocker for 18 hours. It was then buffered with 0.0125 M citrate buffer at pH 5.5 and centrifuged again. Part of the concentrate was inactivated by heating at 100° for 2 minutes, while the balance was used as a source of active enzyme preparation. Various additions were made to the active and inactive levulan preparations, as shown in Table I. The final concentration of levulan in all samples was 22 mg. per ml., representing approximately a 5-fold concentration of the original medium. The activity of the enzyme preparation was roughly tested by allowing it to act in one-sixth of the final concentration on a 6 per cent sucrose solution. In 24

TABLE I

Decomposition of Levulan with Concentrated Enzyme Preparation

Experiment No.	Levulan and enzyme concentrate	Additions	Reducing sugar (as mg. fructose per ml.) produced from levulan in 24 hrs. at 30°
1	Inactivated	None	0
2	"	Glucose	0
3	"	Invertase	1.2
4	"	" fructose	1.1
5	"	" glucose	1.2
6	Active	None	2.6
7	"	Fructose	2.6
8	"	Glucose	2.8
9	"	Invertase	4.0
10	"	" fructose	3.7
11	"	" glucose	6.2
12	"	" " phosphate	6.2

hours at 30°, 2.1 mg. of sucrose were transformed to levulan and glucose and an additional 7.6 mg. hydrolyzed per ml. 0.02 per cent invertase (Wallerstein) was used where indicated, while the sugars were added to make the final concentration 0.043 M. 0.008 M phosphate was added to one tube as NaH_2PO_4-Na_2HPO_4 buffer at pH 5.5. The samples were incubated under toluene in rubber-stoppered test-tubes at 30°.

Determinations of reducing sugar were made with the method of Hassid (10), while the levulan was estimated from the amount of reducing sugar appearing after hydrolysis with 0.3 N HCl for 4 minutes at 100°. The degradation of levulan was followed by measurement of reducing sugar appearing in the mixtures. That this reducing sugar is actually formed from levulan was shown by the fact that in all cases the total reducing value upon acid hydrolysis remained constant throughout the experiment, so that the appearance of sugar was balanced by the disappearance of polysaccharide. It will be seen from Table I that a slow hydrolysis of levulan is effected by the enzyme preparation as well as by the invertase. In combination, the two agents have an approximately additive effect in the absence of glucose. The addition of glucose has no significant effect on the action of either the enzyme preparation or of invertase alone on levulan, but significantly increases the amount of reducing sugar produced from the polysaccharide when both active bacterial enzyme and invertase are allowed to act together.

This may be interpreted as indicating the following reactions.

$$\text{(1)} \quad \text{Levulan} + H_2O \xrightarrow{\text{bacterial enzyme } (a)} \text{fructose (or other products of hydrolysis)}$$

$$\text{(2)} \quad \text{Levulan} + H_2O \xrightarrow{\text{invertase}} \text{fructose (or other products of hydrolysis)}$$

$$\text{(3)} \quad \text{Levulan} + \text{glucose} \xleftrightarrow{\text{bacterial enzyme } (b)} \text{sucrose}$$

$$\text{(4)} \quad \text{Sucrose} + H_2O \xrightarrow{\text{invertase}} \text{fructose} + \text{glucose}$$

Reactions 1, 2, and 4 appear to be irreversible because of the relatively high concentration of water, while the direction of Reaction 3 is normally observed from right to left, and the demonstration of its occurrence from left to right must depend on Reaction 4. Apparently the invertase content of the bacterial enzyme preparations themselves is insufficient to remove the sucrose formed in Reaction 3 rapidly enough to make an appreciable difference between the rates of levulan breakdown with and without glucose.

That the cleavage of levulan proceeds from the ends of the molecule rather than through splitting into levulans of lower molecular weight is indicated by the fact that a rather considerable reducing value is obtained without much visible change in the opalescence or viscosity of the solution. Mild acid hydrolysis, on the other hand, brings about a rapid reduction in the colloidal properties with very slight increases in reducing value.

As might be expected, fructose cannot replace glucose in increasing the rate of levulan breakdown. Other experiments with mannose and galactose in place of glucose were not sufficiently convincing to rule out the possibility of a slight reaction of these sugars with levulan, but indicated that if such a reaction does occur, it is very slow as compared with the one involving glucose.

It is also clear that the addition of phosphate does not increase the rate of the degradations of levulan. This would be fully expected from the observation that added phosphate is unnecessary for its formation. No esterification of inorganic phosphate was ever observed with either sucrose or levulan as substrate for the bacterial enzymes.

Experiments of longer duration than 24 hours were no more convincing than those of short duration. Although a greater amount of reducing sugar is formed if incubation is prolonged, the ratio of excess sugar appearing in the presence of glucose to that produced hydrolytically decreases markedly with time. This is particularly true at temperatures higher than 30°, and is apparently due to the gradual inactivation of the levulan-forming enzyme.

Experiments with Precipitated Enzyme—In preliminary experiments it had been found possible to obtain enzyme preparations from the culture medium which could catalyze the synthesis of levulan from sucrose and were relatively free of preformed polysaccharide. The method of separating the enzymes from the viscous medium was suggested by the successful preparation of dextran-synthesizing enzyme by Hehre (1), and depends on the adsorption of the active principle on a chloroform emulsion, followed by precipitation with alcohol. While the method has not as yet been sufficiently developed to obtain good recovery or very active preparations, it seemed desirable to repeat the observations on the reversibility of levulan synthesis with such material. In addition, the use of these preparations made it possible to test their activity on levulan partly degraded by mild acid hydrolysis.

The source of enzymes was a 48 hour culture of *Bacillus subtilis* grown under conditions already described, except that the concentration of yeast autolysate in the medium was reduced to 2 per cent by volume and 0.05 per cent NH_4Cl was added. 750 ml. of the culture were centrifuged and the supernatant left overnight in the ice box. Ice shavings were then added and the medium was emulsified in 250 ml. lots with 20 ml. amounts of ice-cold chloroform in a Waring blendor. The emulsion was centrifuged lightly and the aqueous supernatant discarded. The pooled chloroform emulsion was washed three times by reemulsification with 600 ml. amounts of ice-cold water and centrifugation as above. The emulsion was then broken up by shaking with 300 ml. of cold 95 per cent alcohol and left for

1 hour in the ice box. It was then centrifuged at high speed, the alcohol drained off and discarded, and the precipitate resuspended in 15 ml. of 0.125 M citrate buffer at pH 5.5. Insoluble material was removed by centrifugation, and the supernatant, which contained only a small amount of levulan, was used as source of enzymes for the experiments. When allowed to act in a 1:10 dilution on a 6 per cent sucrose solution for 24 hours at 30° the preparation effected the conversion of 2.0 mg. of sucrose to levulan and glucose and the hydrolysis of an additional 5.1 mg. of the sugar per ml. 1.5 volumes of this solution were added to 5 volumes of experimental mixtures containing the substrates.

A solution of levulan was prepared from heat-inactivated culture medium centrifuged free of bacteria, concentrated by vacuum distillation, and dialyzed for 24 hours against running distilled water. Part of the levulan

TABLE II

Decomposition of Levulan with Precipitated Enzyme Preparation

Experiment No.	Substrate	Additions*	Reducing sugar (as mg. fructose per ml.) produced from levulan in 18 hrs. at 30°
1	Levulan	None	0.3
2	"	Invertase	1.1
3	"	" glucose	1.9
4	"	" fructose	1.1
5	Degraded levulan	"	2.5
6	" "	" glucose	3.4

was degraded by mild acid hydrolysis with 0.1 N HCl for 60 minutes at room temperature, after which the acid was neutralized with NaOH. Sufficient NaCl was added to the untreated levulan to attain the same salt concentration as in the hydrolyzed material. 26 mg. of the treated and untreated levulan were used as substrate per ml. of solution. The unhydrolyzed levulan possessed a very small reducing value with ferricyanide reagent, while the degraded material gave a value approximating 4 per cent of that obtained on complete hydrolysis.

Sugars and invertase were added in the same concentration as in the previous experiment. Incubation was for 18 hours at 30° under toluene. Table II shows the results of such an experiment.

It will be seen that the precipitated enzymes behave in the same manner as the concentrated culture medium, and that the partially hydrolyzed levulan can act as a substrate at least as well as undegraded polysaccharide. The excess sugar produced in the presence of invertase and glucose (difference between Experiments 2 and 3) is almost 3 times the amount of sugar

produced by hydrolysis alone with the bacterial enzymes (Experiment 1). It is clear that the rate of hydrolysis of the degraded levulan is greater than that of whole levulan, undoubtedly due to the increase in molar concentration of the substrate. The experimental results show but little corresponding increase in the rate of excess sugar production in the presence of glucose. However, the limitations of the method of measuring small changes in reducing value in the presence of relatively large amounts of sugar, and the uncertainty as to the stability of the enzyme and the effect of glucose on invertase activity under the conditions of the experiment, make it impossible to draw any conclusions regarding relative rates of the reverse reaction with hydrolyzed and unhydrolyzed levulan. In other experiments, in which hydrolyzed levulan was added to unprecipitated enzyme concentrates, there was good evidence that the rate of reaction with glucose is increased by approximately the same factor as the rate of hydrolysis by degradation of the polysaccharide.

DISCUSSION

The indirect evidence of the reversibility of levulan synthesis indicates a new biological mechanism for the synthesis of sucrose. It is most probable that, by using a similar approach, the reversible nature of dextran synthesis and a third mechanism of sucrose formation could be shown. The extremely low rate of reaction between levulan and glucose observed in the experiments precludes the possibility of demonstrating the accumulation of sucrose by any but indirect means, unless the rate can be increased enormously.

Although the type of mechanism studied may be of some importance in the biological syntheses of polysaccharides, it seems unlikely that it can be useful for such processes as the transformation of starch to sucrose in plant tissues. The equilibrium appears to be far in favor of the polysaccharide, which is present in relatively low molar concentration owing to its high molecular weight.

Of fundamental interest is the rôle of the glycosidic bond in the transformation of disaccharides to polysaccharides. In the system studied there appears to be an exchange of residues attached to fructose, without the accumulation of an intermediate phosphoric ester. It is, of course, possible that transitory intermediates of unknown nature may occur on the enzyme surface. It seems fair to conclude that the residues of at least some glycosides may play a rôle similar to that of the phosphate group of glucose-1-phosphate in the biological synthesis of complex carbohydrates.

SUMMARY

1. In a system containing levulan, glucose, yeast invertase, and enzymes from the culture medium of *Bacillus subtilis*, evidence of the reversibility

of levulan synthesis and for a new mechanism of sucrose formation was obtained.

2. In addition to the levulan-synthesizing enzyme, the bacterium excretes a hydrolytic enzyme capable of attacking levulan.

3. The enzymatic decompositions of levulan and of levulan partially degraded by mild acid hydrolysis could be demonstrated with enzymes separated from the culture medium by adsorption on a chloroform emulsion, followed by alcohol precipitation.

BIBLIOGRAPHY

1. Hehre, E. J., and Sugg, J. Y., *J. Exp. Med.*, **75**, 339 (1942).
2. Kagan, B. O., Latker, S. N., and Zfasman, E. M., *Biokhimiya*, **7**, 93 (1942).
3. Doudoroff, M., Kaplan, N., and Hassid, W. Z., *J. Biol. Chem.*, **148**, 67 (1943).
4. Doudoroff, M., *J. Biol. Chem.*, **151**, 351 (1943).
5. Hassid, W. Z., Doudoroff, M., and Barker, H. A., *J. Am. Chem. Soc.*, **66**, 1416 (1944).
6. Doudoroff, M., Hassid, W. Z., and Barker, H. A., *Science*, **100**, 315 (1944).
7. Hehre, E. J., *Proc. Soc. Exp. Biol. and Med.*, **54**, 240 (1943).
8. Hestrin, S., and Avinieri-Shapiro, S., *Biochem. J.*, **38**, 2 (1944).
9. Beijerinck, M. W., *Folia microbiol.*, **1**, 377 (1912); Verzamelde Geschriften van M. W. Beijerinck, Delft, **5**, 89 (1922).
10. Hassid, W. Z., *Ind. and Eng. Chem., Anal. Ed.*, **9**, 228 (1937).

STUDIES IN CARBOHYDRATE METABOLISM

V. EFFECTS OF ADRENALIN AND INSULIN UPON GLYCOGENESIS IN RATS*

By DeWITT STETTEN, Jr., and BABETTE V. KLEIN

(*From the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York*)

(Received for publication, May 4, 1945)

In the earlier papers of this series (1–4) studies of the nature and rate of glycogenesis in the liver and in the muscle of rats have been reported. Observations on the uptake of deuterium into glycogen from D_2O in the body fluids have been made with normal rats eating glucose (1) and galactose (4), with previously fasted rats given glucose or lactate (2), and with a diabetic rat receiving glucose (3). In the present paper we report further observations in which the rate and nature of glycogenesis have been influenced by variation of other conditions.

Effect of Adrenalin upon Fasted Rats—Since very little glycogen persists in the liver of a rat after 24 hours of fasting, if the glycogen content of such a liver is made to increase abruptly, the newly deposited glycogen will be minimally contaminated with preexisting glycogen. If this rapid rise in liver glycogen occurs while the body fluids are enriched with D_2O, then the deuterium concentration in the total liver glycogen, minimally diluted by preexisting non-isotopic glycogen, should approach very closely the deuterium concentration of the freshly synthesized product.

It has been pointed out (1) that the highest concentration of deuterium observable under such conditions should lie in the neighborhood of 66 per cent of that in the body water, and that this value would be achieved only if each hydrogen atom in the glycogen were, in the animal, of the same isotopic composition as the hydrogen of the body water. This would require that, at some stage during glycogenesis, each hydrogen atom was either derived from or exchangeable with hydrogen of the body fluids. These conditions were experimentally approximated in the case of liver glycogen obtained from previously fasted rats fed lactate (2). This sample of glycogen contained 57 per cent as high a concentration of deuterium as the body fluids from the same animals.

The administration of adrenalin to previously fasted rats has been shown to result in a decrease in muscle glycogen and an increase in liver glycogen, and it has been proposed that adrenalin stimulates anaerobic glycolysis in

* This work was carried out with the aid of grants from the Nutrition Foundation, Inc., and the Josiah Macy, Jr., Foundation.

muscle with liberation of lactic acid which the liver utilizes for glycogenesis (5). We have argued that if the previously fasted rat, when given adrenalin, actually makes liver glycogen out of lactate, this glycogen, in our type of experiment (Table I), should show the same isotope distribution as the glycogen from the livers of the previously fasted rats fed lactate.

A comparison of these two experiments is given in Table II. The administration of either lactate by stomach tube or adrenalin subcutaneously to previously fasted rats resulted in a marked increase in the quantity of liver glycogen over the fasting level. The deuterium concentrations of these two samples were essentially identical, 57.0 and 56.2 per cent respectively of the concentrations in the body water. This agreement is precisely what would be expected from the hypothesis of Cori (5) and is taken as confirmation of his proposal that the extra liver glycogen that appears when a fasted rat is given adrenalin is synthesized from blood lactate. Too little glycogen remained in the muscles of these rats to permit satisfactory isolation.

Effect of Insulin upon Previously Fasted Rats—When glucose was fed by stomach tube to previously fasted rats containing D_2O in their body fluids, the glycogen which was deposited in large amounts in the liver was not as rich in deuterium as was the liver glycogen in the corresponding experiment in which lactate was fed (2). This type of glycogenesis, with the introduction of appreciably less than the theoretically maximal amount of deuterium, has also been encountered in well nourished rats fed glucose (1) or galactose (4) at a dietary level of 60 per cent. The explanation must lie in the synthesis of at least a portion of the glycogen by the more or less direct utilization of the non-isotopic dietary hexose without preliminary degradation to fragments with completely and readily exchangeable hydrogen. The simplest assumption is that direct conjugation of dietary glucose molecules to glycogen takes place.

In similar studies conducted on a rat rendered diabetic with alloxan it was observed that the glycogen, both in liver and in muscle, though reduced in quantity, was richer in deuterium than in the corresponding non-diabetic animals (3). This finding was taken to mean that the type of glycogenesis favored by insulin was the direct conjugation of glucose, which involves the introduction of little or no isotope, and that the process persisting in the diabetic animal was glycogenesis from smaller fragments involving the incorporation of much deuterium from the body fluids.

Two experiments have now been completed in which the converse effect, the effect of extra insulin upon normal animals, has been studied. Insulin in appropriate dosage was injected into previously fasted rats with the simultaneous administration of glucose on the one hand and lactate on the other. In neither instance was there any increase in quantity of liver glyco-

gen over fasting levels, a finding which, in the case of the lactate feeding, is in accord with the recent report of Kaplan and Greenberg (6). The amounts of liver glycogen recovered were too small to permit of deuterium analysis.

The muscle glycogen in both experiments was markedly increased in quantity over the fasting levels, about 7-fold when glucose was fed and more than 3-fold when lactate was fed. In relation to the tremendous increases in quantity of glycogen in the muscle, the concentrations of deuterium in these samples were quite low. The inference that the extra glycogen deposited in the muscle in response to insulin was very poor in isotope, considered together with the earlier results, confirms the belief

TABLE I

Deuterium Concentrations in Glycogen and Fatty Acids of Previously Fasted Rats Treated with Adrenalin and with Insulin

Six adult female rats in each group were fasted for 24 hours and then given D_2O subcutaneously. 1 hour later insulin or adrenalin was injected and glucose or lactate fed by stomach tube in dosages indicated in the text. The rats were killed 3 hours thereafter.

The results are expressed in atom per cent deuterium.

Treatment	Body water	Liver glycogen	Carcass glycogen	Liver fatty acids	Depot fatty acids
Adrenalin	1.28	0.720		0.016	0.007
Insulin + glucose	1.30		0.276	0.007	0.000
" + lactate	1.19		0.338	0.007	0.006

that the process favored by insulin, the process impeded in diabetes, is the direct utilization of hexose for glycogenesis.

In the diabetic animal a second observed defect was the marked retardation of fatty acid synthesis, lipogenesis (3). We have therefore sought for conditions under which the converse of this effect, stimulation of lipogenesis in response to administered insulin, would be manifest. Whereas increased lipogenesis in the liver might or might not result in an increase in the quantity of fatty acid in the liver, depending on the integrity of the fat transport systems, in our type of experiment it would almost necessarily result in an increase in the deuterium concentration in the liver fatty acids. The failure to find such an increase in the liver fatty acids of the normal rats receiving insulin, as compared with others that received no insulin (Table II), indicates that no great increase in fatty acid synthesis could have occurred in the livers of these animals.

Alloxan-Diabetic Rat on Glucose-Free Diet—We have previously shown that a rat rendered diabetic with alloxan, kept on a high carbohydrate diet,

incorporated deuterium from body water into glycogen of both liver and muscle at an increased rate, indicating active glycogenesis largely from fragments smaller than hexose. The synthesis of fatty acids, on the other

TABLE II

Uptake of Deuterium in Glycogen and in Fatty Acids of Previously Fasted Rats

The data in Table I have been recalculated on the basis of per cent of D in the body water and are here compared with previously published results (2).

Treatment	Liver glycogen		Carcass glycogen		Liver fatty acids	Depot fatty acids
	Weight	D	Weight	D	D	D
	per cent of liver	*per cent of body water*	*per cent of body*	*per cent of body* water*	*per cent of body water*	*per cent of body water*
None*	0.04	25.1	0.011	11.9	0.3	1.2
Lactate*	0.21	57.0	0.012	15.4	1.7	2.2
Adrenalin	0.37	56.2			1.3	0.5
Glucose*	0.82	38.1		14.2	1.2	0.9
Insulin + glucose	0.03		0.074	21.2	0.5	0.0
" + lactate	0.01		0.038	28.4	0.6	0.5

* Boxer and Stetten (2).

TABLE III

Uptake of Deuterium in Glycogen and in Fatty Acids of Diabetic Rats

Adult rats rendered diabetic with alloxan or treated with phlorhizin have been maintained for 56 hours with elevated D_2O concentration in their body fluids. The diets fed contained, on the one hand, 60 per cent of carbohydrate, and on the other, 60 per cent of *dl*-alanine and no carbohydrate.

	Alloxan diabetes,* carbohydrate diet			Alloxan diabetes, alanine diet			Phlorhizin poisoning, carbohydrate diet		
	Weight	D	D	Weight	D	D	Weight	D	D
	gm.	*atom per cent*	*per cent of body water*	*gm.*	*atom per cent*	*per cent of body water*	*gm.*	*atom per cent*	*per cent of body water*
Body water		2.32	100.0		1.69	100.0		1.78	100.0
Liver fatty acids	0.164	0.212	9.1	0.200	0.092	5.4	0.193	0.302	17.0
Depot " "	1.154	0.209	9.0	5.4	0.048	2.8	16.4	0.128	7.2
Liver glycogen	0.124	1.00	43.1	0.067	0.937	55.5	0.093	0.285	16.1
Carcass glycogen	0.290	0.518	22.3	0.255	0.224	13.3	0.233	0.162	9.1

* Stetten and Boxer (3).

hand, was markedly retarded in that the diabetic rat synthesized only about 0.1 gm. of fatty acids daily, whereas the normal rat would synthesize almost 2.0 gm. (3). It is now well established that normal rats on high carbohy-

drate diets synthesize large amounts of fatty acids daily (7) and it is not surprising, therefore, that as "total diabetes" is approached and the metabolism of glucose in general is impaired, the utilization of glucose in lipogenesis should also be retarded.

We have now performed on a diabetic rat a second experiment in which, however, no glucose was added to the diet. In its place 60 per cent of *dl*-alanine, a known glucogenic amino acid (8), was added. When an alloxan-poisoned rat, exhibiting approximately the same degree of glucosuria as that used previously, was placed on the alanine diet, a prompt diminution in glucosuria was noted. The animal was maintained for 56 hours with an elevation of D_2O in its body fluids and the tissues were then investigated precisely as before (3).

Although somewhat more depot fat persisted in this animal than in the earlier one (Table III), the deuterium concentrations in the fatty acids of both liver and depot were extraordinarily low. Calculating as we have previously,

$$\frac{2 \times 5.4 \times 0.200}{100} + \frac{2 \times 2.8 \times 5.4}{100} = 0.324 \text{ gm.}$$

of newly synthesized fatty acids was deposited in 56 hours, or 0.14 gm. per day. This value is close to the value of 0.10 gm. per day deposited by the diabetic rat on a high carbohydrate diet and in contrast to the 1.9 gm. deposited daily by the normal, non-diabetic rat. It may therefore be concluded that, in the rat rendered diabetic with alloxan, lipogenesis is impeded regardless of whether glucose or alanine constitutes the bulk of the diet.

In contrast to the fatty acids, the glycogen samples from this rat were very rich in isotope. The liver glycogen once again is seen to approach the theoretically maximal value, reaching 55.5 per cent of that in the body water. In view of the experience when lactate was fed, it was to be expected that during glycogenesis in rats on a diet composed predominantly of alanine, practically all of the hydrogen atoms should achieve equilibrium with the labeled body water.

After this rat was placed on the alanine diet, the urine contained but little glucose. However, some could be isolated from the pooled specimen for the 56 hours of observation. Employing methods previously described (3), we have calculated the quantity of urinary glucose that was synthesized *in vivo*, and, as shown in Table IV, about 50 per cent of the glucose excreted was thus synthesized from smaller fragments during the period of observation.

Utilization of Dietary Carbohydrate by Phlorhizinized Rat—By way of contrast, a rat was rendered "diabetic" by the daily administration of

phlorhizin and was studied by the same procedures while maintained on a 60 per cent carbohydrate diet. Whereas very high concentrations of urinary glucose were attained, the polyuria seen in alloxan diabetes did not occur, nor did the striking loss of depot fat. The body fluids were enriched with D_2O for 56 hours prior to death, as in the other experiments with diabetic rats.

Despite the glucosuria, this rat analytically resembled closely the normal rat on an equivalent diet. The deuterium concentrations in the fatty acids and glycogen of liver and of carcass were very near to what would be expected in the normal animal, as may be seen by comparison with

TABLE IV

Isotopic Composition of Urinary Glucose

From the urine of rats rendered diabetic with alloxan or treated with phlorhizin and maintained with an elevated D_2O concentration in their body fluids, glucose has been isolated as the pentaacetate and analyzed for D. The urine samples were pooled urine collected during the 56 hours following the initiation of D_2O administration.

Experiment	Urine volume	Urine glucose		D in water	D in glucose		Glucose synthesized	
	cc.	*per cent*	*gm.*	*atom per cent*	*atom per cent*	*per cent of water*	*gm.*	*per cent*
Alloxan diabetes, carbohydrate diet*	284	5.3	14.2	2.00	0.288	14.4	3.6	25.4
Alloxan diabetes, alanine diet	155	0.2	0.3	1.61	0.465	28.8	0.15	49.5
Phlorhizin poisoning, carbohydrate diet	24	13.8	3.3	1.38	0.282	20.4	1.2	35.0

* Stetten and Boxer (3).

interpolated values for 56 hours from our normal data (1). The concentrations of deuterium expected in normal rats on this diet after 56 hours would be about 22 per cent in the liver fatty acids, 23 per cent in the liver glycogen, and 9 per cent in the carcass glycogen. The corresponding values obtained in the phlorhizinized rat (Table III) were 17, 16, and 9 per cent respectively. That fatty acid synthesis may have been somewhat impaired is suggested by the fact that the calculated quantity of freshly synthesized fatty acid deposited,

$$\frac{2 \times 17.0 \times 0.193}{100} + \frac{2 \times 7.2 \times 16.4}{100} = 2.43 \text{ gm.}$$

in 56 hours, or about 1 gm. per day, is less than the corresponding value for the normal animal. The decrease, however, is unimpressive compared with the decrease in fatty acid synthesis seen in the alloxan-diabetic rats.

The urinary glucose of the phlorhizinized rat was also found to contain deuterium. In view of the previously reported findings with galactose (4), we feel reasonably confident in assigning the deuterium in the urinary glucose to *in vivo* synthetic reactions, and on this basis it may be calculated that about 35 per cent of the glucose present in this urine sample was synthesized by the rat. The alloxan-diabetic rat on the same diet synthesized about 25 per cent of its urinary glucose (Table IV).

These figures are taken to mean that both rats synthesized from 25 to 35 per cent as much glucose each day as they derived from their diet. The dietary and the synthetic glucose were mixed and a portion of the mixture appeared in the urine. As these rats consumed daily 12 to 15 gm. of dietary glucose, a synthesis of 3 to 5 gm. of glucose daily must be postulated. This synthesis we believe to be essentially normal gluconeogenesis. Excessive gluconeogenesis, "overproduction," cannot be invoked to account for the glucosuria of alloxan diabetes unless one is willing to assume a similar "overproduction" in the phlorhizinized rat. For such an assumption there is, we believe, no foundation.

Lipogenesis in Hypoinsulinism—The apparent failure of the alloxan-diabetic rat to deposit newly synthesized fatty acids in the fats of its body is reminiscent of our experience with the thiamine-deficient rat (9). Here also it could be shown by the deuterium technique that the ability to synthesize fatty acids from the ingredients of a high carbohydrate diet is largely lost, and this finding fits nicely into the concept of the thiamine-sparing action of fat and the well substantiated improvement in condition of thiamine-deficient animals following the isocaloric substitution of fat for carbohydrate (10).

The recent observation of Burn, Lewis, and Kelsey (11) that alloxan-diabetic rats fared better on high fat than on low fat diets suggests that fat exerts a sparing action upon insulin, much as it does upon thiamine. This insulin-sparing action is of course subject to the limitation of ketosis, but to this complication rats appear to be unusually resistant. It is noteworthy, however, that both in thiamine deficiency and in hypoinsulinism increase of the fat content of the diet at the expense of carbohydrate is followed by improvement and, from our evidence, both thiamine and insulin appear to be essential for the optimal synthesis of fatty acids by rats on a high carbohydrate diet.

EXPERIMENTAL

Three experiments have been carried out on fasted rats, a group of six rats being employed in each experiment. The time schedule of prior experiments (2) was strictly adhered to. Adult female rats of the Sherman strain, previously maintained on stock diet and weighing on the average

200 gm., were deprived of all food for 24 hours, water being allowed *ad libitum*. Each rat was then given a subcutaneous injection of 99.5 per cent D_2O containing 0.9 per cent of NaCl, 1 cc. per 100 gm. of body weight. 1 hour thereafter each rat of the first group was given 0.1 cc. of an adrenalin solution, 1:5000, per 100 gm. of body weight. Each rat of the second group was given 250 mg. of glucose in 1.25 cc. of water by stomach tube immediately followed by the subcutaneous injection of 10 units of insulin per 100 gm. of body weight. To each rat of the third group, 400 mg. of *dl*-lactic acid, half neutralized with NaOH and dissolved in 1.25 cc., were fed by stomach tube, followed by the injection of 4 units of insulin per 100 gm. of body weight. 4 hours after the administration of D_2O, 3 hours after the treatment and tube feedings, the rats were killed by a blow on the head, the livers removed, the carcasses eviscerated, and the livers and carcasses worked up for glycogen and fatty acids as previously described (2).

Each of three male adult rats was given a single subcutaneous injection of alloxan monohydrate, 20 mg. per 100 gm. of body weight. The diet was the 60 per cent corn-starch diet that had previously been employed with alloxan-diabetic rats (3), and, as in the earlier experiment, the drinking water was replaced by 5 per cent glucose solution for the first 24 hours after administration of alloxan. Two of the rats died in 2 days, apparently not in severe ketosis. The surviving animal lost about 10 per cent of his initial body weight in 2 days but thereafter the weight remained constant at 347 ± 7 gm. Food and water were allowed *ad libitum*, and 3 to 7 gm. of glucose were found daily in a urine volume of 100 to 150 cc. A faint test for acetone was present on the 2nd day, but thereafter no ketonuria was detected.

On the 6th day the diet was changed to one comprising 60 parts of *dl*-alanine, 22 parts of casein (Labco, vitamin-free), and 6 parts each of yeast powder, salt mixture (12), and roughage (Celluration). An immediate decrease in the glucosuria was noted. The following day 99.5 per cent D_2O was injected, 1 cc. per 100 gm. of body weight, and the drinking water was replaced by 2.0 per cent D_2O. 56 hours later the rat was killed and the tissues analyzed as before. The total urine volume during this 56 hour interval was 155 cc. and contained only 0.3 gm. of glucose. This was isolated as the osazone for deuterium analysis.

One adult male rat, maintained on a 60 per cent corn-starch diet (3), was given phlorhizin by the daily injection of 0.01 gm. of phlorhizin in sesame oil per 100 gm. of body weight. The daily urine volume averaged 7 cc. and contained 0.7 to 1.7 gm. of glucose. After approximately constant weight had been attained, D_2O was injected and administered in the drinking water over a period of 56 hours, as in the previous experiment, with the one difference that the drinking water supplied contained 2.5 per cent

of D_2O. The urine collected over this period contained 3.3 gm. of glucose, which was isolated as the pentaacetate (3). The remainder of the procedure was precisely as in the previous experiment.

The evidence accumulated thus far for the non-exchangeable nature of the carbon-bound hydrogen atoms of carbohydrate under a variety of conditions has been summarized in an earlier paper (4). We now have additional evidence indicating the stability of these carbon to hydrogen bonds in acid solution. A sample of glycogen containing 0.320 atom per cent deuterium was obtained from the liver of a rabbit which had received D_2O. A portion of this material was heated to 100° for 1 hour in N H_2SO_4, and, after removal of the acid, glucose was isolated from the hydrolysis mixture as the pentaacetate. This product contained 0.168 atom per cent D.

Assuming the deuterium initially present to have been distributed in the 66 per cent of stable positions, the actual concentration in these positions may be estimated as $0.320 \times 100/66 = 0.49$ atom per cent. The deuterium concentration in the 7 stable positions of the glucose may be computed from the analysis of the pentaacetate, $0.168 \times 22/7 = 0.53$ atom per cent. From this it may be concluded that none of the deuterium present in the glycogen sample isolated was lost by exchange during the treatment with hot acid.

SUMMARY

The formation of glycogen in the previously fasted rat in response to adrenalin and to insulin has been studied by the isotope technique. The data secured support the hypothesis that the glycogen appearing in the liver of a fasted rat after injection of adrenalin is formed from blood lactate. The glycogen appearing in the muscle of rats after administration of insulin is apparently formed largely from hexose directly.

The failure of lipogenesis in the alloxan-diabetic rat has been confirmed. The synthesis of fatty acids has been shown to be markedly impaired, whether glucose or alanine constitutes the bulk of the diet. No comparable impairment in lipogenesis was found to occur in the phlorhizinized rat.

The quantity of glucose synthesized *in vivo* in the alloxan-diabetic rat is no greater than the corresponding quantity in the phlorhizinized rat. The actual quantity in an adult male rat has been estimated to lie in the neighborhood of 3 to 5 gm. per day.

BIBLIOGRAPHY

1. Stetten, D., Jr., and Boxer, G. E., *J. Biol. Chem.*, **155**, 231 (1944).
2. Boxer, G. E., and Stetten, D., Jr., *J. Biol. Chem.*, **155**, 237 (1944).
3. Stetten, D., Jr., and Boxer, G. E., *J. Biol. Chem.*, **156**, 271 (1944).
4. Stetten, D., Jr., *J. Biol. Chem.*, **159**, 123 (1945).
5. Cori, C. F., and Cori, G. T., *J. Biol. Chem.*, **79**, 309 (1928).

6. Kaplan, N. O., and Greenberg, D. M., *J. Biol. Chem.*, **156**, 553 (1944).
7. Bernhard, K., and Bullet, F., *Helv. chim. acta*, **26**, 1185 (1943).
8. Ringer, A. J., and Lusk, G., *Z. physiol. Chem.*, **66**, 106 (1910).
9. Boxer, G. E., and Stetten, D., Jr., *J. Biol. Chem.*, **153**, 607 (1944).
10. Arnold, A., and Elvehjem, C. A., *Am. J. Physiol.*, **126**, 289 (1939). Stirn, F. E., Arnold, A., and Elvehjem, C. A., *J. Nutr.*, **17**, 485 (1939).
11. Burn, J. H., Lewis, T. H. C., and Kelsey, F. D., *Brit. Med. J.*, **2**, 752 (1944).
12. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **15**, 311 (1913).

THE OCCURRENCE OF DICHOLESTERYL ETHER IN THE SPINAL CORD OF THE OX (BOS TAURUS)

By HENRYK SILBERMAN* and SOFIA SILBERMAN-MARTYNCEWA

(Received for publication, May 14, 1945)

Dicholesteryl ether was prepared for the first time from cholesterol by dehydration with anhydrous copper sulfate by Mauthner and Suida (1) in 1896. Bills and McDonald (2) (see also for a comprehensive survey of previous literature) prepared dicholesteryl ether by heating cholesterol with different catalysts and they also recorded its chemical and physical constants in detail.

A search of the literature reveals that there is no record of the separation of dicholesteryl ether from natural sources.

In the preparation of cholesterol from animal brains it was noticed that when spinal cord was substituted for brain as the starting material, the cholesterol recovered could not be purified by repeated crystallizations from boiling alcohol, as could easily be accomplished in the case of the cholesterol recovered from brain. Even after eight to ten crystallizations there remained in the cholesterol an impurity which caused a sintering of the material at a temperature of about 137–140°, and after the material was melted the liquid remained turbid even at 160–170°. A thorough investigation of the cholesterol prepared from spinal cord revealed that the impurity consisted of dicholesteryl ether which was identified by analysis and its physical constants.

The spinal cord of young cattle was dried on flat trays for 8 to 12 hours at 65–75° under reduced pressure of 30 to 40 mm. of Hg. The completely dried material was extracted continuously for 12 hours with hot alcohol. The solvent was removed by evaporation under reduced pressure. The residue was repeatedly extracted with warm acetone. After removal of the solvent the remaining cholesterol was recrystallized twice from boiling alcohol. To separate the dicholesteryl ether from the bulk of cholesterol the white crystalline material was repeatedly treated with hot acetone. The residue, a white microcrystalline powder, was further purified by extraction with ether to remove the last traces of cholesterol, and then crystallized by dissolving in hot benzene, treating with charcoal, filtering, and precipitating by adding absolute alcohol. The separated white crystalline powder was filtered off after cooling and washed with cold alcohol. A further crop of the material was recovered by evaporating the benzene-

* Ermington, New South Wales, Sydney, Australia.

alcohol solvent mixture, and replacing the evaporated benzene by alcohol. The substance was dried over P_2O_5 *in vacuo* at 70–75°. It melts (after sintering at 190–200°) at 205–209°. It is soluble in boiling benzene, toluene, and alcohol, slightly soluble in cold benzene or toluene, and very slightly soluble in cold alcohol, warm ether, and acetone.

18.3 mg. of the substance (dried over P_2O_5 at 70° and 12 mm. of Hg) gave 57.6 mg. of CO_2 and 20.6 mg. of H_2O.

$C_{54}H_{90}O$. Calculated, C 85.93, H 11.93; found, C 85.81, H 12.5

For further identification the tetrabromide (3) was prepared by dissolving 1 gm. of the dicholesteryl ether in 60 cc. of dry $CHCl_3$, and adding 0.45 gm. of bromine dissolved in 5 cc. of the same solvent. After standing for 6 hours, the clear cherry-red solution was evaporated at ordinary temperature under a vacuum to remove the solvent and the excess bromine. The residue, a yellow resin, was dissolved in 4 to 5 cc. of $CHCl_3$ and precipitated by adding methanol. The white flocculent precipitate was filtered after standing 24 hours and washed with methanol. The material when heated in a capillary tube (after drying *in vacuo*) turns yellow-brown at 110–130° and melts with decomposition at 166–168° (Levin, m.p. 164–166°, with decomposition).

SUMMARY

The quantity of the dicholesteryl ether isolated from the spinal cord amounts to about 1.5 to 2 per cent of the weight of the dry starting material.

BIBLIOGRAPHY

1. Mauthner, J., and Suida, W., *Monatsh. Chem.*, **17**, 29 (1896).
2. Bills, C. E., and McDonald, F. G., *J. Biol. Chem.*, **72**, 1 (1927).
3. Levin, R. H., *J. Am. Chem. Soc.*, **65**, 627 (1943).

THE DIETARY PRODUCTION OF FATTY LIVERS RESISTANT TO THE ACTION OF CHOLINE

BY M. L. MACFARLAND AND E. W. MCHENRY

(*From the School of Hygiene, University of Toronto, Toronto, Canada*)

(Received for publication, May 14, 1945)

In 1940 it was reported from this laboratory (1) that the administration to rats of a beef liver fraction, in conjunction with several B vitamins, caused the production of fatty livers, high in cholesterol, resistant to the lipotropic action of choline, but preventable by lipocaic. Inositol was found to be lipotropic for the fatty liver thus produced (2). Subsequently it was stated (3) that the liver fraction could be replaced by biotin; at that time, however, practically all of the investigation was carried out with a relatively crude solution of biotin, pure biotin being available for one group of rats only. Pure biotin produced fatty livers containing large amounts of cholesterol and they appeared to resemble the ones caused by the beef liver fraction or by impure solutions of biotin. Consequently, this kind of fatty liver (resistant to choline) has been referred to as the "biotin" type. Recently we have had an opportunity to investigate more thoroughly the production of this kind of fatty liver. It would appear that pure biotin produces a fatty liver only partially resistant to choline and that the beef liver fraction contains a substance, other than biotin, which causes a more complete resistance to choline.

Methods

Rats were employed as test animals. The strain, age, and care were the same as previously described (4). To deplete the rats of their stores of B vitamins and of fat, they were maintained for 3 weeks on a fat-free, B vitamin-free basal diet (4). At the end of this period body weight and body fat had diminished markedly. During the following week various combinations of supplements were supplied to different groups. Thiamine, riboflavin, pyridoxine, calcium pantothenate, and biotin were given by subcutaneous injection. Supplements other than these were mixed with the food. The amounts of various supplements supplied per rat per day were as follows: thiamine hydrochloride (Merck) 25 γ, riboflavin (Merck) 25 γ, pyridoxine hydrochloride (Merck) 40 γ, calcium pantothenate (Merck) 100 γ, choline hydrochloride (Merck) 10 or 20 mg. as indicated, biotin (S. M. A.) 5 γ, inositol (S. M. A.) 10 or 20 mg. as indicated, and beef liver fraction (Connaught Laboratories) 2 cc. The liver fraction was similar to that used previously (5).

TABLE I

Effects of Liver Fraction and of Biotin upon Action of Lipotropic Agents

Rat group No.	Treatment during test wk.	Body weight change in test wk	Liver weight	Crude fatty acids							Total cholesterol				
				Liver			Carcass			Total	Liver		Carcass		Total
						Iodine No.			Iodine No.						
		gm.	*gm.*	*mg. per liver*	*per cent*		*mg. per carcass*	*per cent*		*mg. per rat*	*mg. per liver*	*per cent*	*mg. per carcass*	*per cent*	*mg. per rat*
231	Depleted; no supplements		3.4	131	3.8	74	1200	1.9	77	1,331	8	0.23	190	0.30	198
226	B vitamins, liver fraction	+40	7.9	1585	20.1	63	8344	8.3	57	9,929	80	1.01	311	0.31	391
227	Same as Group 226 + 10 mg. choline	+42	8.0	1573	19.7	56	9344	9.0	64	10,917	92	1.15	280	0.27	372
229	" " " 226 + 20 " "	+40	8.3	1424	17.2	58	8352	8.4	65	9,776	79	0.95	259	0.26	338
228	" " " 226 + 10 " inositol	+42	5.9	268	4.5	69	9072	8.7	62	9,340	24	0.40	250	0.24	274
230	" " " 226 + 20 " "	+34	5.7	217	3.8	82	8054	8.2	67	8,271	19	0.33	246	0.25	265
232	" " " 226 + 10 " choline, 10 mg. inositol	+35	6.1	276	4.5	72	8309	8.6	64	8,585	21	0.34	249	0.26	270
252	B vitamins, biotin	+22	7.3	1827	25.0	59	5862	6.8	64	7,689	50	0.68	226	0.26	276
254	Same as Group 252 + 10 mg. choline	+23	5.4	537	10.0	66	7315	8.1	62	7,852	34	0.62	256	0.28	290
256	" " " 252 + 20 " "	+23	5.3	488	9.2	62	6718	7.5	65	7,206	33	0.62	251	0.28	284
253	" " " 252 + 10 " inositol	+21	5.7	875	15.5	62	5960	6.8	65	6,835	39	0.69	199	0.23	238
255	" " " 252 + 20 " "	+24	5.6	595	10.7	62	7084	7.8	63	7,679	33	0.60	221	0.24	254
257	" " " 252 + 10 " choline, 10 mg. inositol	+23	4.8	197	4.1	71	6912	7.5	64	7,109	14	0.30	227	0.25	241

The rats were anesthetized with nembutal, the livers removed, and the total crude fatty acids in the livers and bodies were determined by methods previously published (4, 6). The total cholesterol content of the livers and bodies was measured by means of the procedure of Schoenheimer and Sperry (7) and the iodine numbers of the crude fatty acids were determined by the method of Yasuda (8). All reported results are averages for groups of ten rats.

Results

The effects of a beef liver fraction in causing fatty livers in rats have been repeatedly confirmed in this laboratory. For the present purpose it was advisable to demonstrate the effect again under conditions comparable to those in which biotin was used. The experiment with biotin was repeated twice and entirely similar results were secured on each occasion; for brevity only one set of results is given. Table I shows the data secured by the use of beef liver fraction and with biotin.

DISCUSSION

As in a number of similar experiments, the administration of the beef liver fraction, in conjunction with several B vitamins, caused the production of acutely fatty livers in rats maintained on a high carbohydrate, fat-free ration. There was evidence of synthesis of large amounts of fat and of considerable cholesterol. Choline, at the dosage levels used, caused negligible changes in the concentration and in the absolute amounts of fat and of cholesterol in the liver. Given in the same amounts as used for choline, inositol produced marked reductions in liver fat and cholesterol. The effect of a combination of choline and inositol is similar to that produced by the same amount of inositol alone. It is obvious that inositol is effective in preventing the fatty liver caused by the beef liver fraction, while choline is not.

In the case of three similar experiments, one of which has been reported above, biotin has produced acutely fatty livers, but the cholesterol is less than when liver fraction was supplied. Both choline and inositol are partially effective as lipotropic agents for the fatty livers produced with biotin and a combination of the two is much more effective than like amounts of the two substances given separately. As judged by the action of these lipotropic agents, the fatty liver caused by biotin is definitely different from that obtained with the beef liver fraction. The term "biotin fatty liver" should be restricted and is not applicable, as we had assumed previously (9), to fatty livers caused by the beef liver fraction.

In an earlier paper (2) attention was drawn to the action of inositol in causing a marked decrease in liver cholesterol. Such is the case when liver

fraction is supplied but in the biotin experiments both choline and inositol exerted an effect upon the cholesterol content of the liver. Reference has been made (1) to the synthesis of cholesterol which occurs in experiments such as the present one. It is not clear whether a particular constituent of the diet promotes the synthesis of cholesterol or whether cholesterol is formed as an accompaniment to fat synthesis. It is also doubtful whether a particular lipotropic agent causes the removal of cholesterol from the liver or whether the amount of cholesterol is reduced because it is removed with fatty acids. In the biotin experiment the second alternative seems plausible. Beveridge and Lucas (10) reported that, under the conditions used by them, choline produced greater decreases in liver cholesterol than did inositol. It would appear that liver cholesterol is reduced when the total amount of fat is diminished and that a lipotropic agent is effective for cholesterol when it definitely reduces the amount of fatty acids in the liver.

An explanation for the difference in the actions of the two lipotropic factors cannot be supplied at present. Some of the conditions which alter the response were indicated in a report from this laboratory in 1943 (11). Choline was found to be effective for "thiamine fatty livers," while inositol was not; inositol was active for "biotin fatty livers" (a term then incorrectly used), while choline was inactive. It would appear that inositol becomes effective when several B vitamins are supplied. Recently Beveridge and Lucas have reported (10) that the inclusion of corn oil in the diet prevents inositol from exerting a lipotropic effect. It was suggested in a review from this laboratory (12) that choline will combine with certain fatty acids, while inositol selects quite different ones. It should be noted that the iodine number of the crude fatty acids in the liver is somewhat greater when liver fraction is given than when biotin is supplied. When inositol is fully active (as in Rat Group 230), the iodine number of the liver fatty acids is definitely increased. This can hardly be taken to mean that inositol has combined with saturated fatty acids and caused their removal from the liver, since there appears to be an increase in the iodine number of liver fatty acids whenever a lipotropic agent has been active and has produced a turnover of fatty acids. A comparison of the fatty acids present in the livers of rats with and without a supply of beef liver fraction (13) showed that the liver fraction causes a decrease in the concentration of saturated fatty acids and an increase in unsaturated ones, particularly of oleic acid. A similar study has not yet been made on the livers of rats given biotin.

The constituent of the liver fraction which causes the alteration in the response to the lipotropic factors is unknown at present. The liver fraction is known to contain most of the B vitamins as well as folic acid and other

substances. On the basis of previous work (11) many of these can be eliminated as causative agents (thiamine, riboflavin, pyridoxine, pantothenic acid). It should be pointed out that the liver fraction contains no cholesterol. Attempts to identify the active constituent are in progress.

SUMMARY

The administration of a beef liver fraction to rats maintained on a high carbohydrate, fat-free diet causes the production of fatty livers resistant to choline, but the liver fat can be reduced to low levels by supplying inositol. The use of biotin in place of the liver fraction causes fatty livers which are partially responsive to either lipotropic agent and completely prevented by a conjoint supply of choline and inositol.

This investigation was made possible by grants from the Division of Natural Sciences of the Rockefeller Foundation and from the Connaught Laboratories, University of Toronto.

BIBLIOGRAPHY

1. McHenry, E. W., and Gavin, G., *Science*, **91**, 171 (1940).
2. Gavin, G., and McHenry, E. W., *J. Biol. Chem.*, **139**, 485 (1941).
3. Gavin, G., and McHenry, E. W., *J. Biol. Chem.*, **141**, 619 (1941).
4. Gavin, G., and McHenry, E. W., *J. Biol. Chem.*, **132**, 41 (1940).
5. McHenry, E. W., and Gavin, G., *J. Biol. Chem.*, **134**, 683 (1940).
6. McHenry, E. W., and Gavin, G., *J. Biol. Chem.*, **125**, 653 (1938).
7. Schoenheimer, R., and Sperry, W. M., *J. Biol. Chem.*, **106**, 745 (1934).
8. Yasuda, M., *J. Biol. Chem.*, **94**, 401 (1931-32).
9. McHenry, E. W., Biological symposia, Lancaster, **5**, 177 (1941).
10. Beveridge, J. M. R., and Lucas, C. C., *J. Biol. Chem.*, **157**, 311 (1945).
11. Gavin, G., Patterson, J. M., and McHenry, E. W., *J. Biol. Chem.*, **148**, 275 (1943).
12. McHenry, E. W., and Patterson, J. M., *Physiol. Rev.*, **24**, 128 (1944).
13. Longenecker, H. E., Gavin, G., and McHenry, E. W., *J. Biol. Chem.*, **139**, 611 (1941).

TRYPTOPHANE UTILIZATION AND SYNTHESIS BY STRAINS OF LACTOBACILLUS ARABINOSUS

By LEMUEL D. WRIGHT AND HELEN R. SKEGGS

(*From the Nutritional Laboratories, Department of Pharmacology, Medical-Research Division, Sharp and Dohme, Inc., Glenolden*)

(Received for publication, April 9, 1945)

The amino acid requirements of *Lactobacillus arabinosus* 17-5 have been the subject of several recent studies (1). Although tryptophane is usually considered an essential amino acid for the growth of this organism, Snell has shown (2) that *Lactobacillus arabinosus* may utilize indole or anthranilic acid in lieu of tryptophane. These observations are in accord with the view that the synthesis of tryptophane in nature proceeds through anthranilic acid and indole as intermediates (2, 3).

We have been able to produce strains of *Lactobacillus arabinosus* that no longer require tryptophane and are capable of seemingly indefinite growth on transfer in a completely synthetic medium entirely lacking in tryptophane or its known precursors. Although capable of excellent growth in such a medium, the altered strains do, however, respond to tryptophane and its intermediates in a unique manner. These observations are reported because of their relationship to the microbiological assay of amino acids and to bacterial metabolism in general.

EXPERIMENTAL

The compositions of the media employed are given in Table I. In certain lots of casein hydrolysate, the tryptophane was not completely destroyed by acid hydrolysis. The addition of formaldehyde (1 ml. of formalin to 50 gm. of casein) to the hydrolyzing mixture resulted in a product entirely free from tryptophane, as evidenced by microbiological assay with the parent strain of *Lactobacillus arabinosus*.

The techniques employed throughout this study were those commonly used in microbiological assays with lactic acid bacteria (5).

The altered strains of *Lactobacillus arabinosus* were produced by transferring the parent organism daily in Medium *a* to which 2 γ of tryptophane per 10 ml. had been added. This medium is seriously deficient in tryptophane and consequently at first permitted only poor growth. After transfer in such a deficient medium for about a week, strains were developed that were capable of growing well in the presence of the low level of tryptophane. Growth then readily resulted upon transfer to Medium *a* containing no added tryptophane. The altered strains were maintained by frequent

transfer in Medium *a* or *b*. Medium *b*, consisting only of purified amino acids as a source of nitrogen, was equally effective in promoting growth of the altered strains and thus excluded the possibility that growth in unsupplemented Medium *a* was due to a utilization by the altered strains of possible breakdown products of tryptophane produced during acid hydrolysis of the casein. Medium *a* was consequently used in the subsequent experiments with the altered strains because of its greater convenience.

TABLE I

Composition of Media Employed *

Component	Medium *a*	Medium *b*
Acid-hydrolyzed casein (4), *gm*	10	
l-Cystine, *mg*	200	400
Glucose, *gm*	40	20
Sodium acetate, $3H_2O$, *gm*	20	12
Adenine, *mg*	10	100
Guanine, *mg*	10	100
Xanthine, *mg*	10	100
Uracil, *mg*	10	100
dl-Alanine, *l*-arginine, *dl*-aspartic acid, *l*-glutamic acid, glycine, *dl*-histidine, *l*-hydroxyproline, *dl*-isoleucine, *l*-leucine, *l*-lysine, *dl*-methionine, *dl*-phenylalanine, *l*-proline, *dl*-serine, *dl*-threonine, *l*-tyrosine, *dl*-valine, *mg. of each*		400
Thiamine chloride, *mg*	2	2
Riboflavin, *mg*	2	2
Calcium pantothenate, *mg*	2	2
Nicotinic acid, *mg*	2	2
Pyridoxine hydrochloride, *mg*	4	4
p-Aminobenzoic acid, *mg*	1	1
Biotin, γ	10	10
Salts A (5), *ml*	10	10
Salts B (5), *ml*	10	10
pH 6.6–6.8		

* The quantities given are those employed in preparing 1 liter of double strength medium. The figures in parentheses are bibliographical references.

Bacterial growth has been expressed quantitatively as turbidity readings obtained with the Klett-Summerson photoelectric colorimeter, 540 μ filter, after 24 hours of growth, or as ml. of 0.1 N acid produced in each 10 ml. of culture after 72 hours of growth.

The responses to indole and anthranilic acid are expressed in terms of tryptophane. Consequently the curves in each figure are comparable with each other on a molar basis.

Fig. 1 illustrates a typical response of the unaltered *Lactobacillus arab-*

inosus to increasing amounts of tryptophane, indole, or anthranilic acid after approximately 24 hours of growth.

Fig. 2 is representative of the growth response of the altered strains of *Lactobacillus arabinosus* to increasing amounts of tryptophane, indole, or anthranilic acid after 24 hours of growth.

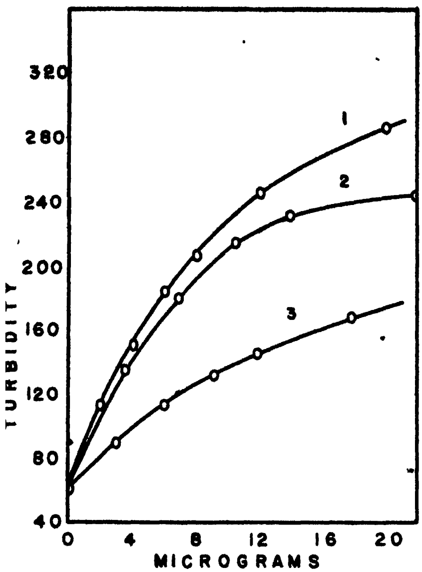


FIG. 1

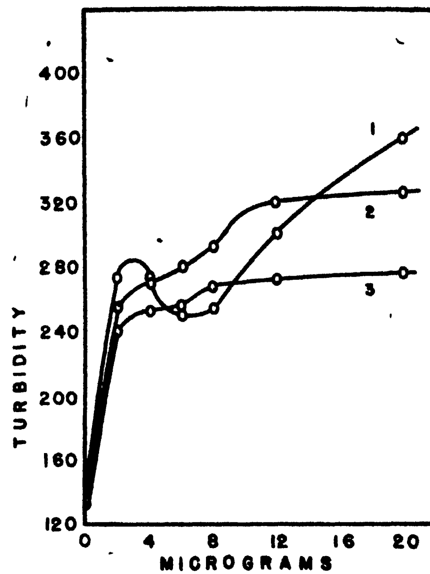


FIG. 2

FIG. 1. Response of the parent strain of *Lactobacillus arabinosus* to (Curve 1) tryptophane, (Curve 2) indole, and (Curve 3) anthranilic acid after 24 hours of growth. Medium *a* was used.

FIG. 2. Response of an altered strain of *Lactobacillus arabinosus* to (Curve 1) tryptophane, (Curve 2) indole, and (Curve 3) anthranilic acid after 24 hours of growth. Medium *a* was used.

In Fig. 3 is given the response of the parent strain and an altered strain of *Lactobacillus arabinosus* to tryptophane, indole, or anthranilic acid after approximately 72 hours of growth. In this experiment the test was prepared in duplicate. One series was seeded with the parent strain while the second series was seeded with an inoculum of approximately equal density of an altered strain.

DISCUSSION

The ability of the altered strains of *Lactobacillus arabinosus* to grow without tryptophane in the medium probably is due to an increased synthetic ability of these strains rather than to any decreased demand for

tryptophane. If this is the case, the growth that occurs when the altered strains are grown in the presence of tryptophane, or known precursors, is the summation of (1) that resulting when tryptophane or other known intermediates are utilized to supply the requirement for tryptophane and (2) that resulting from the synthesis of tryptophane from unknown precursors. Provided the inocula with the two organisms are equal, any increase in growth of the altered strains over that obtained on the

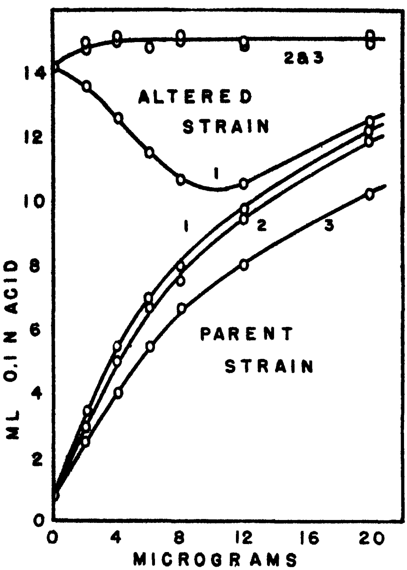


FIG. 3

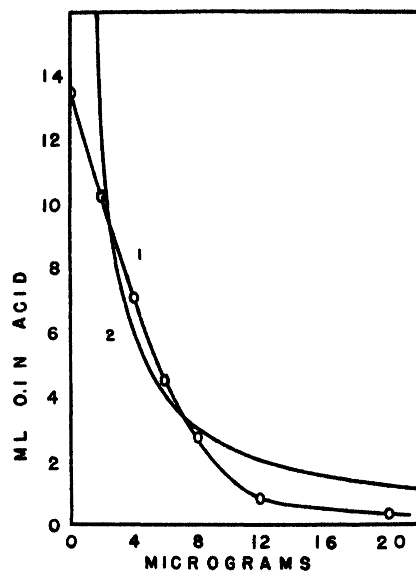


FIG. 4

FIG. 3. Response of the parent strain and an altered strain of *Lactobacillus arabinosus* to (Curve 1) tryptophane, (Curve 2) indole, and (Curve 3) anthranilic acid after 72 hours of growth. Medium *a* was used.

FIG. 4. Amount of growth resulting from the bacterial synthesis of tryptophane after 72 hours of growth as a function of added tryptophane; (Curve 1) calculated curve, (Curve 2) curve for $y = a/x$.

same medium with the parent strain may be taken as a measure of the amount of growth permitted by the tryptophane synthesis of the altered organism. Such a subtraction has been made from the tryptophane data of Fig. 3 and plotted against the amount of added tryptophane as the abscissa (Fig. 4). Within the range of about 2 to 20 γ per tube of added tryptophane the curve approximates that of $y = a/x$. Obviously it must deviate from such a curve at low values of x, since bacterial growth is arrested by products of metabolism and by the depletion of nutrients.

Ultimately the amount of tryptophane synthesized in the presence of

added tryptophane is inversely proportional to that present in the medium. In effect, then, the addition of tryptophane to the medium in which the altered strain is grown results in the organism depending more on the added tryptophane than on its ability to synthesize it.

Indole and anthranilic acid show less effect in influencing growth. In some experiments the rate of growth actually was greater in the presence of certain concentrations of indole or anthranilic acid than in the presence of an equivalent amount of tryptophane (see Fig. 2).

The 24 hour growth data with the altered strain (Fig. 2) have shown a maximum at 2 to 4 γ of added tryptophane per tube. Apparently a certain concentration of tryptophane permits an initial population of organisms to be built up, which, for the first 24 hours, outgrow those whose growth is retarded either by the absence of tryptophane or by the increased tendency of the organism to utilize added tryptophane rather than to synthesize it.

The behavior of the altered strains of *Lactobacillus arabinosus* in response to tryptophane may be due to an effect whereby the presence of tryptophane inhibits its synthesis by mass action. Possibly the synthesis of tryptophane is due to the existence of adaptive enzymes that are produced only in direct proportion to the immediate tryptophane demand.

Additional experiments have shown that the altered strains of *Lactobacillus arabinosus* in the absence of tryptophane are indistinguishable from the parent strain in their requirement for leucine, isoleucine, valine, cystine, glutamic acid, and pantothenic acid. It would appear, therefore, that the ability to synthesize tryptophane by the altered strains of *Lactobacillus arabinosus* is not accompanied by an increased capacity to synthesize other amino acids essential for the parent organism. The requirement for methionine is more pronounced, however, with the altered strains than with the parent strain.

In the use of *Lactobacillus arabinosus*, or other organisms, in microbiological assays, the possibility should not be overlooked that the nutritive requirements of the organism may be influenced materially by methods of handling. The requirement of *Lactobacillus arabinosus* for *p*-aminobenzoic acid appears quite variable (6) and the present studies have demonstrated the ease with which the requirement for a single amino acid may be modified.

SUMMARY

1. Strains of *Lactobacillus arabinosus* that do not require tryptophane, indole, or anthranilic acid in the medium readily can be developed.

2. The response of these strains to tryptophane indicates that, when supplied with tryptophane, the organism produces tryptophane in an amount inversely proportional to that added.

BIBLIOGRAPHY

1. Shankman, S., *J. Biol. Chem.*, **150**, 305 (1943). Kuiken, K. A., Norman, W. H., Lyman, C. M., and Hale, F., *Science*, **98**, 266 (1943). Hegsted, D. M., *J. Biol. Chem.*, **152**, 193 (1944).
2. Snell, E. E., *Arch. Biochem.*, **2**, 389 (1943).
3. Tatum, E. L., Bonner, D., and Beadle, G. W., *Arch. Biochem.*, **3**, 477 (1944).
4. Williams, R. J., and coworkers, *Univ. Texas Pub.*, *No. 4137*, 82 (1941).
5. Snell, E. E., and Wright, L. D., *J. Biol. Chem.*, **139**, 675 (1941).
6. Williams, R. J., Eppright, M. A., Cunningham, E., and Mills, C. A., *Arch. Biochem.*, **5**, 299 (1944).

THE VERATRINE ALKALOIDS

XXIII. THE RING SYSTEM OF RUBIJERVINE AND ISORUBIJERVINE

By WALTER A. JACOBS AND LYMAN C. CRAIG

(*From the Laboratories of The Rockefeller Institute for Medical Research, New York*)

(Received for publication, April 30, 1945)

In a previous communication (1), it has been shown that rubijervine parallels the behavior of solanidine in certain reactions which are best explained on the assumption that Ring B is 6-membered, as in the sterols. The possibility of a 5-membered ring in other alkaloids of the veratrine group was suggested in earlier communications (2) to afford a basis for possible interpretations which had been given to certain dehydrogenation and oxidation products of these alkaloids, and to explain the failure to obtain methylcyclopentenophenanthrene or other phenanthrene derivatives from any one of them. It remained, therefore, of importance to study further the remaining alkaloids of the group, or at least such significant individual alkaloids as would be representative of the different categories. In connection with the rubijervine category, we wish to report our further experience with isorubijervine, and also additional data obtained with rubijervine itself.

Our investigation of the isorubijervine isolated from commercial samples of *Veratrum album* and *Veratrum viride* has shown the identity of the alkaloids from both sources. As previously reported, isorubijervine yields a digitonide. The alkaloid recovered from the digitonide has proved to be indistinguishable from the alkaloid before precipitation with digitonin; $[\alpha]_D^{25} = +8.5°$ in absolute ethanol. As in the case of rubijervine, isorubijervine yielded with copper powder a ketone, *isorubijervone*, which was found to be isomeric with rubijervone. It was characterized by its *oxime*. The same substance was obtained by the Oppenauer method with aluminum tertiary butylate (3). On reduction with aluminum isopropylate, the ketone was readily transformed to the hydroxyl derivative which was presumably a mixture of epimers. From this mixture, on treatment with digitonin, a digitonide was obtained from which *alloisorubijervine* was separated; $[\alpha]_D^{25} = +63°$ in chloroform solution. This substance gave with trichloroacetic acid a gradually developing, deep purple color. The same color, and of similar intensity, was obtained from the mixture before separation with digitonin.

Isorubijervine thus paralleled the behavior of rubijervine in yielding an isomer which gives the Rosenheim color reaction, and which can be explained on the same basis as the conversion of cholesterol to allocholesterol;

i.e., by the shift of the double bond from the original 5,6 position to the 4,5 position during the formation of the intermediate isorubijervone. This interpretation would indicate, therefore, that isorubijervine does not carry an angular methyl group on carbon atom 5, and implies that its Ring B is 6-membered.

That the non-nitrogenous portion of isorubijervine possesses the normal sterol ring system is given further support by its behavior on dehydrogenation with selenium, although a high temperature reaction. However, contrary to the usual sterols, no evidence of the production of methylcyclopentenophenanthrene could be obtained. Instead, a hydrocarbon $C_{17}H_{14}$ was isolated as a principal product. In its melting point of 135–136° and other properties, it closely agreed with what has been recorded for 1,2-cyclopentenophenanthrene (4). This identity was supported by the study of its picrate, which melted at 134–135°, and of its trinitrobenzene derivative, which melted at 165–166°.

Although 1,2-cyclopentenophenanthrene[1] was considered in the early discussions concerning the exact identity of Diels' hydrocarbon, it was eventually established beyond question that the latter must be 3′-methyl-1,2-cyclopentenophenanthrene. The actual formation of cyclopentenophenanthrene as a dehydrogenation product from any natural substance has not been recorded, as far as we have been able to find. Its production is of special interest in the case of isorubijervine, since its formation would indicate some structural feature of the alkaloid which interferes with the normal shift of the angular methyl on carbon atom 13, to replace the side chain on carbon atom 17 during dehydrogenation. Either the stereochemical configurations are such as to block this shift, or the second hydroxyl group of the alkaloid is situated on or in the vicinity of the angular methyl group or carbon atom 17. The attempt will now be made to check such possibilities in other ways. At any rate, the formation of cyclopentenophenanthrene can be regarded as evidence for the presence of this ring structure in isorubijervine which is just as good as the production of Diels' hydrocarbon in the case of the other sterol derivatives.

A further study of rubijervine has since shown that the rubijervone previously described can be obtained also by the Oppenauer reaction with acetone and aluminum tertiary butylate. A repetition of the reduction of the ketone with aluminum isopropylate with a larger amount of material has led to the separation of *allorubijervine* and *epiallorubijervine* from the mixture. It has now been found that the previously described substance appears to be epiallorubijervine, since it does not give a sparingly soluble digitonide. Its rotation is $[\alpha]_D^{26} = +63°$ in absolute alcohol. From the most soluble fraction of the reaction mixture, a digitonide was obtained,

[1] A review of this subject is very well given by L. F. Fieser (5).

and from this a more soluble isomer was isolated which can be interpreted as allorubijervine; $[\alpha]_D = +40°$ in absolute alcohol. Both isomers gradually developed pronounced purple colors with trichloroacetic acid.

The trivial names provisionally adopted for the various substances in which the prefix, *allo*, has been used, were suggested by the analogy to *allo*cholesterol. This is a matter of convenience, although perhaps somewhat confusing in view of the conventional use of *allo* to distinguish the allocholanic from the cholanic series, due to the configuration on carbon atom 5. When the structural and configurational correlation of the veratrine bases to one another and to solanidine and the sterols has once been completed, it will be possible to assign more rational names based on those adopted for the parent unsubstituted saturated hexacyclic tertiary and pentacyclic secondary bases.

EXPERIMENTAL

Isorubijervine and Digitonin—0.16 gm. of isorubijervine from *Veratrum viride* was dissolved in 24 cc. of 95 per cent alcohol and treated with 48 cc. of warm 1 per cent digitonin solution. The digitonide soon began to crystallize and after 22 hours was collected with 95 per cent alcohol. 0.515 gm. was obtained; calculated, 0.64 gm. The substance was dissolved in 10 cc. of pyridine and precipitated with several volumes of ether. The precipitate was collected with a good volume of ether. The ether solution was repeatedly extracted with water to remove pyridine and, after drying, was concentrated. All ether was finally replaced with 95 per cent alcohol and on careful dilution and heating the alkaloid separated as needles. 87 mg. were obtained. The substance as previously recorded tended to separate at first as needles which contained solvent and melted at 218° or as anhydrous small prisms which melted at 241–244°.

$$[\alpha]_D^{25} = +8.5° \ (c = 0.94 \text{ in absolute ethanol})$$

$C_{27}H_{43}O_2N$. Calculated, C 78.38, H 10.49; found, C 78.61, H 10.30

0.11 gm. of isorubijervine from *Veratrum album* gave relatively the same yield of digitonide, 0.35 gm. The isorubijervine recovered from the latter was indistinguishable in properties from the above alkaloid.

$$[\alpha]_D^{25} = +9.4° \ (c = 1.01 \text{ in absolute ethanol})$$

Found, C 78.42, H 10.40

Isorubijervone—A mixture of 1 gm. of isorubijervine (from *Veratrum viride*) with 5 gm. of copper powder was gradually heated at ordinary pressure in a sublimation apparatus after removal of air with CO_2. The heating occurred gradually from 100–200° during the first 15 minutes for removal of solvent and then from 200–290° for an additional 15 minutes.

After cooling, the apparatus was exhausted to about 0.1 mm. and the temperature was gradually raised in the course of an hour to 290°, during which a red-colored sublimate was collected. After removal from the condenser with chloroform and concentration, 0.9 gm. of residue was obtained which crystallized readily from alcohol as lustrous masses of 4-sided almost rectangular leaflets. 0.5 gm. was obtained in the first crop. The substance did not exhibit a sharp melting point. After recrystallization from alcohol following preliminary sintering and softening, it melted above 250°, but crystals persisted to 255°.

$$[\alpha]_D^{27} = +111° \ (c = 1.07 \text{ in pyridine})$$

$C_{27}H_{41}O_2N$. Calculated, C 78.77, H 10.05; found, C 78.60, H 9.72

Isorubijervine from *Veratrum album* yielded a substance with identical properties.

$$[\alpha]_D^{27} = +111° \ (c = 0.98 \text{ in pyridine})$$

Found, C 78.60, H 9.99

0.2 gm. of isorubijervine from *Veratrum viride* was refluxed for 4.5 hours in a mixture of 3 cc. of acetone, 6 cc. of benzene, and 5 cc. of a benzene solution of 0.35 gm. of aluminum tertiary butylate. The reaction mixture was treated with excess dilute alkali and extracted with chloroform. The extract after removal of solvent yielded a crystalline residue which in turn gave a first fraction of 0.11 gm. of colorless leaflets from alcohol. In melting point and other properties, the substance was indistinguishable from that obtained with copper.

$$[\alpha]_D^{28} = +109.5° \ (c = 0.86 \text{ in pyridine})$$

Found, C 78.46, H 9.95

The *oxime* was readily obtained from the keto base in dilute aqueous acetic acid solution with hydroxylamine hydrochloride and sodium acetate. A salt of the oxime soon separated. After liberation from the latter with alkali and extraction with chloroform, the oxime was obtained from 95 per cent alcohol as small prisms which softened to a melt with slow effervescence at 250–254° after preliminary discoloration.

$C_{27}H_{42}O_2N_2$. Calculated, C 75.99, H 9.93; found, C 75.75, H 10.00

Alloisorubijervine—0.25 gm. of isorubijervone was refluxed for 22 hours with aluminum isopropylate prepared from 1 gm. of aluminum foil and worked up essentially as previously described in the case of allorubijervine. The ether extracts after concentration yielded 0.14 gm. of nearly rectangular plates or broad needles, which from the melting point was an obvious mixture. Under the microscope it sintered above 200° and showed signs of melting, especially above 225°, and then progressively melted but solid

persisted up to 242°. It gave a gradually developing deep purple color with trichloroacetic acid.

$C_{27}H_{43}O_2N$. Calculated, C 78.38, H 10.49; found, C 78.51, H 10.19

80 mg. of the substance were dissolved in 12 cc. of 95 per cent alcohol and treated with 33 cc. of a 1 per cent digitonin solution in 90 per cent alcohol. The digitonide very slowly separated. After 2 days the collected material weighed 0.116 gm. The filtrate after several days further standing gradually yielded an additional 80 mg. The first fraction was dissolved in 5 cc. of dry pyridine and the digitonin was precipitated with ether. After filtration and washing with ether, the filtrate was further diluted with ether and then repeatedly extracted with water. The ether solution yielded long stout often rhombic platelets or masses with serrated edges. After recrystallization from an alcohol-ether mixture, the substance melted at 250–251° after preliminary sintering.

$C_{27}H_{43}O_2N$. Calculated, C 78.38, H 10.49; found, C 78.03, H 10.46
$[\alpha]_D^{25} = +63°$ ($c = 0.95$ in chloroform)

This substance gave the characteristic gradually developing deep purple color in trichloroacetic acid.

Rubijervone—0.2 gm. of dried rubijervine in 7 cc. of benzene was treated with 0.35 gm. of aluminum tertiary butylate and 3 cc. of acetone, and the mixture was refluxed for 6.5 hours. The reaction mixture was treated with dilute alkali and extracted with chloroform. The latter yielded on concentration a residue which crystallized as needles from 95 per cent alcohol. The yield of the first fraction was 64 mg. 46 mg. of less pure additional material were obtained from the mother liquor.

After recrystallization from alcohol, the substance softened under the microscope at 203° and gradually melted at 205–209°.

$[\alpha]_D^{30} = +100°$ ($c = 0.93$ in 95% ethanol)
$C_{27}H_{41}O_2N$. Calculated, C 78.77, H 10.05; found, C 78.86, H 9.87; C 78.84, H 10.07

This substance proved to be indistinguishable in properties from the substance previously obtained by the copper powder method (1).

The melting point of the latter has since been found to be 205–208° after preliminary softening above 202°, and gave no depression with the above substance. Its rotation has also been found to be

$[\alpha]_D^{30} = +98°$ ($c = 0.94$ in 95% ethanol)

Epiallorubijervine—The reduction of rubijervone was repeated essentially as previously given, except with a larger amount of substance, 0.88 gm. On attempting to extract the reaction product with ether, crystallization at once occurred. The ether suspension of crystals was separated

from the alkaline aqueous phase and washed with water. The crystals which proved to be very sparingly soluble were collected with ether and amounted to 0.3 gm. As previously reported, it gave a deep purple color with trichloroacetic acid. After recrystallization from 95 per cent alcohol, it gradually melted under the microscope at 228–231° after preliminary sintering above 222°.

$$[\alpha]_D^{25} = +63° \ (c = 1.00 \text{ in absolute ethanol})$$

This substance yielded no precipitate with 1 per cent digitonin solution even after several weeks standing.

Allorubijervine—The ether filtrate from the sparingly soluble epiallorubijervine was dried over sodium sulfate and concentrated to smaller volume. A second fraction of 69 mg. of substance was obtained which melted at 212–215° after preliminary softening. This was followed by a third fraction of 26 mg. of still less pure material which gradually softened to a melt at 208–213° after preliminary sintering. When the mother liquor from the last fraction was concentrated to about 5 to 10 cc., it deposited rather gelatinous material. The whole mixture was therefore dried *in vacuo* as such. In this manner a crude fraction of about 0.5 gm. was obtained.

This crude fraction was dissolved in 50 cc. of 95 per cent alcohol and mixed with a solution of 1.1 gm. of digitonin in 100 cc. of 90 per cent alcohol. Since after 24 hours a slight precipitate had formed, the mixture was allowed to stand for 5 days during which the deposition appreciably increased. The collected digitonide weighed 0.35 gm.

This material was decomposed with pyridine and ether. The ether after removal of pyridine left a residue on concentration which was dissolved in 1 to 2 cc. of 95 per cent alcohol. On rubbing, crystallization of minute delicate needles occurred. On addition of more alcohol and a few drops of water, this fraction somewhat increased on standing and was collected with 70 per cent alcohol. 26 mg. were obtained which appeared to be a mixture, since the melting point suggested contamination with the above epimer. On careful further dilution the mother liquor yielded a larger fraction (40 mg.) of long delicate needles which were collected with 50 per cent alcohol. After recrystallization from dilute alcohol, the substance formed broad, flat needles which gradually lost their transparency under the microscope above 140–150° and then melted at 176–178°.

$$[\alpha]_D^{25} = +40° \ (c = 0.97 \text{ in absolute ethanol})$$

For analysis it was dried *in vacuo* at 110°.

$C_{27}H_{43}O_2N \cdot H_2O$. Calculated, H_2O 4.17; found, 3.94
Anhydrous substance, $C_{27}H_{43}O_2N$. Calculated, C 78.38, H 10.49; found, C 78.60, H 10.62

With trichloroacetic acid it gave gradually a deep purple color indistinguishable from that of the above epimer.

Dehydrogenation of Isorubijervine—A mixture of 10 gm. of isorubijervine (from *Veratrum album*) and 30 gm. of selenium was heated for 2 hours in a bath at 340–345° after displacement of air with nitrogen. A highly colored distillate was collected and consisted of basic material, mostly the methylethyl pyridine which has now been obtained from all of this group of alkaloids. The undistilled residue was powdered and extracted thoroughly with ether. This extract on concentration left a residue of 6.5 gm. which was redissolved in ether and extracted with 20 cc. of 10 per cent HCl. The latter was reserved for some future study. The ether phase was then extracted in turn with dilute alkali, which yielded but an inappreciable

TABLE I

Fractionation of Hydrocarbon Mixture

Fraction No.	Bath temperature	Column temperature	Micro melting point	Analysis	
				C	H
	°C.	°C.	°C.	*per cent*	*per cent*
1	190	140			
2	190	120			
3	200	150		90.44	9.69
4	200	150			
5	205	160			
6	205	160	100–123		
7	210	165	100–123	93.11	6.83
8	215	165			
9	225	170	90–120	93.12	6.87
10	250	180			
11	255	210			
12	255	230			

amount of any phenolic material. The ether solution which remained gave on concentration 3.1 gm. of neutral material.

This fraction was dissolved in 50 cc. of benzene and passed through a column of 80 gm. of active alumina. The alumina was in turn eluted with three 50 cc. portions of benzene which on subsequent concentration yielded respectively 1.4, 1.3, and 0.1 gm. of residue. These fractions were combined for fractionation in a 24 cm. Craig column at 0.2 mm. pressure. The course of the fractionation is recorded in Table I. Fraction 1 and 2 each approximated 50 mg. and from Fraction 3 the weights approximated 125–150 mg. The undistilled residue was about 0.5 gm. Fractions 5 to 10 were crystalline. Fraction 10 was somewhat colored, due to contamination with selenium.

110 mg. of Fraction 7, when recrystallized from ether, separated as rosettes of stout needles. After collection at $-18°$, 68 mg. of substance were obtained which melted at 134–136°. On recrystallization from ether, this was sharpened somewhat to 135–136°.

$C_{17}H_{14}$. Calculated, C 93.53, H 6.47; found, C 93.64, H 6.66

Fraction 9 yielded 84 mg. of needles which melted at 131–134°. This material was used for the following derivatives.

30 mg. of hydrocarbon were treated with an equivalent of picric acid in a small volume of acetone. The complex crystallized nicely from the concentrated mixture at $-18°$, but separated in crystals of two apparent shades, one much paler than the other, due possibly to differing crystal solvent content. From acetone-ether it separated in pale orange prisms or needles which melted at 134–135°.

$C_{17}H_{14}\cdot C_6H_3O_7N_3$. Calculated, C 61.72, H 3.83; found, C 61.48, H 3.97

30 mg. of hydrocarbon and 25 mg. of 1,3,5-trinitrobenzene yielded 42 mg. of the complex from acetone solution. Recrystallized from acetone, it formed bundles or rosettes of long needles which melted at 165–166°.

$C_{17}H_{14}\cdot C_6H_3O_6N_3$. Calculated, C 64.01, H 3.97; found, C 64.16, H 3.80

SUMMARY

Isorubijervine has been transformed through its ketone isorubijervone to a mixture from which alloisorubijervine was isolated over its digitonide. Rubijervine was similarly transformed through rubijervone to a mixture of epimers from which allorubijervine and epiallorubijervine were separated. All of these substances give the Rosenheim color reaction of allocholesterol to be explained by a Δ^4 double bond and which implies that Ring B is 6-membered. A unique product of the dehydrogenation of isorubijervine has been found to be 1,2-cyclopentenophenanthrene. A normal sterol ring system of the non-nitrogenous portion of rubijervine and isorubijervine is therefore indicated.

All analytical results reported here were obtained by Mr. D. Rigakos.

BIBLIOGRAPHY

1. Jacobs, W. A., and Craig, L. C., *J. Biol. Chem.*, **152**, 641 (1944).
2. Craig, L. C., and Jacobs, W. A., *J. Biol. Chem.*, **141**, 253 (1941); and earlier papers.
3. Oppenauer, R. V., *Rec. trav. chim. Pays-Bas et Belgique*, **56**, 137 (1937).
4. Ruzicka, L., Ehmann, L., Goldberg, M. W., and Hosli, H., *Helv. chim. acta*, **16**, 833 (1933). Kon, G. A. R., *J. Chem. Soc.*, 1081 (1933).
5. Fieser, L. F., The chemistry of natural products related to phenanthrene, New York, 160 (1937).

DISTRIBUTION OF CATHEPTIC ENZYMES IN THE HOG KIDNEY*

By PAUL C. ZAMECNIK and MARY L. STEPHENSON

(From the Medical Laboratories of the Collis P. Huntington Memorial Hospital of Harvard University, at the Massachusetts General Hospital, Boston)

(Received for publication, May 1, 1945)

The term cathepsin was introduced by Willstätter and Bamann (1) to characterize an intracellular proteinase of animal origin active at weakly acid pH. The presence of a number of catheptic enzymes in animal tissues has been demonstrated in Bergmann's laboratory (2–6) by means of synthetic peptide substrates. The present experiments were undertaken to find out whether these catheptic enzymes differed in activity in histologically distinct portions of the same organ.

The two regions selected for comparison were the outer portion of the renal cortex and the medullary papillae, as diagrammed in Fig. 1. The catheptic activity of the metabolically active cortex, composed of nephrons responsible for filtration, secretion, and absorption, was found to be consistently higher than was the catheptic activity of the collecting tubules which make up the medullary papillae.

EXPERIMENTAL[1]

The rate of hydrolysis was determined by the method of Grassmann and Heyde (7). The composition of the test solution was as follows: 1.0 cc. of 0.1 M substrate (0.12 M for *dl*-leucylglycine), 0.8 cc. of 0.1 M enzyme-buffer solution, 0.2 cc. of 0.4 M cysteine, and 0.01 cc. of toluene. Incubation was carried out in a volumetric flask at 40° in the presence of air. Initial and final pH determinations were made with a glass electrode assembly. The solution of cysteine was freshly prepared before use.

There was a considerable amount of autolysis when extractions were made with water, and appreciable autolysis with 30 per cent glycerol. No autolysis was present with 50 per cent glycerol. In these experiments, therefore, the most reproducible results were obtained with extraction of enzyme from dry tissue powder by means of a 50 per cent glycerol-0.1 M citrate buffer solution. With each set of experiments autolysis controls were carried out.

* This is Reprint No. 603 of the Cancer Commission of Harvard University.

[1] The substrates benzoyl-*l*-argininamide, carbobenzoxy-*l*-glutamyl-*l*-tyrosine, and carbobenzoxyglycyl-*l*-phenylalanine were obtained through the courtesy of the late Dr. Max Bergmann, and *dl*-leucylglycine through the courtesy of Dr. M. S. Dunn.

Nitrogen determinations were made according to the micro-Kjeldahl method of Wagner (8). In order to avoid excessive foaming and charring of the glycerol extracts in the digestion procedure, a very low flame was used until the initial foaming had ceased, after which the flame was increased until the charred mixture became clear. Glycerol extracts took longer to clear than aqueous extracts. Duplicates checked when blow pipettes were used. Total nitrogens of aliquots of 0.9 per cent sodium chloride tissue extract agreed whether the aliquots were digested in the presence of added glycerol or simply in the presence of 0.9 per cent sodium chloride. Glycerol did not, therefore, interfere with the complete digestion of the protein. Non-protein nitrogen filtrates were obtained by precipitation of the protein in a 10 per cent trichloroacetic acid solution.

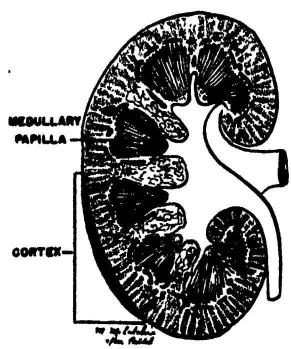


FIG. 1. Diagram of kidney, showing in black the regions selected for enzymatic study.

Ammonium sulfate and acetone purification procedures performed on aliquots of the same dried powder did not give reproducible activity constants.[2]

Preparation of Enzymes

2 pounds of fresh hog kidneys, packed in ice, were obtained from a slaughter-house. The outer portion of the cortices was cut off in strips 2 to 3 mm. thick and 6 to 8 cm. long, and the medullary papillae were carefully dissected away from the remainder of the medulla. In the longitudinally cut kidney the medullary papillae stood out as distinctly redder areas than the remainder of the medulla, which blended into the cortical region. Since these papillae protruded into the renal pelvis, they could easily be dissected out by means of a sharp scalpel. This procedure was carried out at 2.5°. It was impossible to free the medullary papillae

[2] The authors are indebted to Mr. Lester Tobin for assistance in these experiments.

completely from adjacent connective tissue, but as much as possible was removed. The tissues were dried according to the Flosdorf-Mudd (9) technique for 24 hours. The dried tissues were ground to a fine powder (the medullary papillae were sieved to remove a few bits of connective tissue which could not be well ground) and were stored over phosphorus pentoxide in a vacuum desiccator at 2.5°. The rate of inactivation of enzyme under these conditions was slight during a storage period of 2

TABLE I

Hydrolysis of Certain Synthetic Substrates by Glycerol Extracts of Hog Kidney Cortex and Medulla

Substrate	Type of enzyme tested for*	$C \times 10^4$		
		Cortex	Medullary papillae	Cortex / Medulla
Carbobenzoxy-*l*-glutamyl-*l*-tyrosine	Pepsinase	22	4.7	4.7
Carbobenzoxyglycyl-*l*-phenylalanine	Carboxypeptidase	12	2.8	4.3
Benzoyl-*l*-argininamide	Trypsinase†‡	11	3.9	2.8
dl-Leucylglycine	Aminopeptidase†	16	3.0	5.3

Cortex = 2.40 mg. of protein nitrogen per cc. of test solution; medulla = 2.44 mg. of protein nitrogen per cc. of test solution; pH range, 5.06 to 5.22; initial and final pH values constant within 0.05 pH unit in all cases. Citrate buffer to 0.04 M, cysteine 0.04 M, substrate 0.05 M (*dl*-leucylglycine 0.06 M). Temperature 40°, toluene 0.5 per cent. The reaction followed first order kinetics; the average of three closely agreeing readings is recorded in the table. Times chosen to give substrate hydrolysis range from 20 to 70 per cent. Rate of hydrolysis of *dl*-leucylglycine calculated on the basis of splitting of the *l* form. $K = 1/t \log a/(a - x)$. $C = K$ per mg. of protein nitrogen per cc. of test solution.

* The enzyme classification is that suggested by Bergmann (6).

† There is the possibility of some carboxypeptidase activity contributing to the hydrolysis of these substrates.

‡ The term "tryptase" or "tryptic endopeptidase" might be a better choice of terminology for this enzyme, whose action is similar to that of trypsin. Likewise, "peptase" or "peptic endopeptidase" might be preferable to "pepsinase," since the enzyme in question does not split pepsin but rather resembles it in specificity requirements.

permitting a number of determinations on aliquots to be made. The strips of cortex yielded 86 gm. of pale brown powder, whereas 3.3 gm. of reddish powder were obtained from the medullary papillae. The ratio of dry weight to wet weight for the cortex was 20.0 per cent and for the medulla 20.4 per cent.

Extracts of cortex and medullary papillae were prepared in the same way. 1200 mg. of the dried tissue were ground up in a mortar for 4 minutes with 12 cc. of 50 per cent glycerol-0.1 M citrate buffer, pH 5. The solution

was centrifuged for 5 minutes at 2400 R.P.M. and was then filtered through a thin layer of Pyrex glass wool. The filtrate was kept in ice water and its enzymatic activity tested within 2 hours. The extract of kidney cortex contained 7.55 mg. of total nitrogen and 6.01 mg. of protein nitrogen per cc., while the medulla extract yielded 7.90 mg. of total nitrogen and 6.11 mg. of protein nitrogen per cc. The protein nitrogen concentrations of the extracts of cortex and medulla were therefore approximately equal, which eliminates the possibility that differences in enzymatic activity in the two regions may be accounted for by some anomalous relationship between rate constant and enzyme concentration, such as that encountered by Irving *et al.* (10).

Aliquots of the dried tissue, extracted and tested at intervals during a 2 week period, repeatedly gave approximately the same enzymatic activity. As a test of the reproducibility of the methods, twelve separate 500 mg. aliquots of dried renal cortex were extracted and tested for activity toward benzoyl-*l*-argininamide at pH 5 in the presence of added cysteine. The results were as follows, expressed as $C^3 \times 10^{-4}$: 9.7, 9.4, 10.3, 11.8, 8.7, 13.0, 10.3, 11.9, 10.2, 8.4, 10.0, 8.1.[3] The arithmetic mean was 10.2 with the standard error of the mean = 0.43 (11).

Two other batches of kidneys were worked up and gave results similar to those summarized in Table I.

DISCUSSION

The activity of catheptic enzymes was found to be consistently higher in the hog kidney cortex than in the medullary papillae, as shown in Table I, and to exceed the limit of error of the methods used. The difference in the activity ratio of individual enzymes between cortex and medulla ranged from 2.8 to 5.3. More detailed work would be necessary, however, to prove what this latter point suggests, that an individual catheptic enzyme may vary independently of others in different regions of a single organ.

Previous work (4) has demonstrated the presence in the hog kidney of at least four separate catheptic enzymes, characterized and classified according to their specific activities on certain peptide substrates. The same types of key substrates have been used in the present experiments to compare the catheptic enzymes in two distinct regions of the hog kidney.

A histological check was made on aliquots of the tissue selected for study. It should be pointed out that, whereas there is relatively little interstitial connective tissue in the cortex, there is considerably more in the medullary papillae. The enzymatic differences found, therefore, represent this difference in the amount of connective tissue in the two regions as well as the difference between the cortical nephrons and the papillary collecting ducts.

[3] See the explanation below Table I.

SUMMARY

The activity of catheptic enzymes present in the hog kidney has been found to be consistently higher in the cortical than in the medullary region. Lyophilization of fresh tissue and extraction of the dried tissue powder with 50 per cent glycerol-0.1 M citrate buffer at pH 5 provided a satisfactory method of preparing extracts for these studies.

BIBLIOGRAPHY

1. Willstätter, R., and Bamann, E., *Z. physiol. Chem.*, **180**, 127 (1929).
2. Fruton, J. S., and Bergmann, M., *J. Biol. Chem.*, **130**, 19 (1939).
3. Fruton, J. S., Irving, G. W., Jr., and Bergmann, M., *J. Biol. Chem.*, **138**, 249 (1941).
4. Fruton, J. S., Irving, G. W., Jr., and Bergmann, M., *J. Biol. Chem.*, **141**, 763 (1941).
5. Fruton, J. S., *J. Biol. Chem.*, **146**, 463 (1942).
6. Bergmann, M., in Nord, F. F., and Werkman, C. H., Advances in enzymology and related subjects, New York, **2**, 49 (1942).
7. Grassmann, W., and Heyde, W., *Z. physiol. Chem.*, **183**, 32 (1929).
8. Wagner, E. C., *Ind. and Eng. Chem., Anal. Ed.*, **12**, 771 (1940).
9. Flosdorf, E. W., and Mudd, S., *J. Immunol.*, **29**, 389 (1935).
10. Irving, G. W., Jr., Fruton, J. S., and Bergmann, M., *J. Biol. Chem.*, **138**, 231 (1941).
11. Arkin, H., and Colton, R. R., An outline of statistical methods, New York, 4th edition, 126 (1939).

THE MICROBIOLOGICAL ASSAY OF VITAMIN B_c CONJUGATE

By O. D. BIRD, BETTY BRESSLER, RAYMOND A. BROWN, C. J. CAMPBELL, and A. D. EMMETT

(*From the Research Laboratories of Parke, Davis and Company, Detroit*)

(Received for publication, May 3, 1945)

Coincident with work leading to the isolation of vitamin B_c from liver in this laboratory (1), other sources of the vitamin were being investigated. Very early it was observed that only part of the vitamin B_c indicated by chick assays to be present in yeasts and yeast extracts could be accounted for by microbiological assays with *Lactobacillus casei*. This suggested that part of the vitamin present in these substances was in a bound form which was ineffective as a growth stimulant for this organism. Since that time we have extensively studied enzymatic procedures for releasing vitamin B_c from the bound form in which it apparently exists in natural materials, in order to be able accurately to assay them microbiologically. This is a summary of some of the results of this study.

In an earlier report from this laboratory (2) it was indicated that concentrates from yeast which were active in curing anemia in chicks, but only slightly active on *Lactobacillus casei*, became strongly active on *Lactobacillus casei* following enzymatic digestion. Welch and Wright (3) have pointed out the possible existence in milk of a microbiologically inactive folic acid-like material possessing vitamin activity in the rat. Totter, Mims, and Day (4) reported that incubation with fresh rat liver increased the folic acid content of dried brewers' yeast as measured by microbiological assay. Later these workers (5) described the preparation of a fraction from rat liver which was effective in producing a microbiologically active factor from inactive material. Mallory *et al.* (6) reported the concentration of a fraction from yeast which became active microbiologically after treatment with an enzyme preparation. They pointed out a possible correlation between this factor, vitamin M, vitamin B_c conjugate, and a factor in yeast which is antagonistic to the effects of succinylsulfathiazole in the diet of rats. Laskowski, Mims, and Day (7) have recently succeeded in purifying to a considerable degree an enzyme which is active in this conversion.

EXPERIMENTAL

The enzymatic digestion procedure of Cheldelin *et al.* (8), employing taka-diastase and papain, proved to be ineffective in liberating vitamin B_c from its conjugate. However, it was found that either taka-diastase or clarase was partially effective when used in high dilutions. The activity of

these enzyme preparations varied greatly among different lots. Some samples of these products were almost entirely inactive.

Certain animal organs, as well as other natural materials, proved to be more effective. Bird *et al.* have recently noted the occurrence of an enzyme, widely distributed in nature, which formed vitamin B_c from its conjugate (9). In our earlier work various tissues were employed, either ground while fresh, or as a powder prepared by acetone desiccation and milling.

Examples of the action of some of these crude enzyme preparations upon a concentrate of vitamin B_c conjugate from yeast are shown in Table I. The samples were incubated with the enzyme preparations under toluene in small Erlenmeyer flasks at approximately pH 4.5. They were then auto-

TABLE I

Effectiveness of Different Sources of Enzyme in Releasing Vitamin B_c from Its Conjugate

Enzyme source	Enzyme preparation per cc. substrate	Substrate dilution*	Incubation time at 37°	Vitamin B_c found	
				In enzyme (blank)	In substrate (less blank)
	gm.		*hrs.*	γ	γ *per cc.*
Control (no enzyme)					1.8
Fresh beef heart	1.0	1:10	48	0	1.8
" " spleen	1.0	1:10	48	0.15	8.4
Clarase	0.5	1:20,000	48	4.15	8.7
Desiccated hog liver	0.08	1:10	72	0.55	2.6
" " "	0.20	1:10	72	1.30	9.0
" " "	0.50	1:10	72	3.25	13.0
Fresh hog liver	1.0	1:1,000	48	3.5	16.4
Desiccated hog kidney	0.30	1:10	48	0.75	27.0
" " intestine	0.20	1:10	72	0.10	26.3

* A concentrate of vitamin B_c conjugate from yeast.

claved briefly, centrifuged, and the supernatant assayed with *Lactobacillus casei* by a modification of the method of Mitchell and Snell (10), crystalline vitamin B_c being used as the standard. The medium was the same as that described in the published procedure except that the pyridoxine hydrochloride content was increased from 200 γ to 1000 γ per liter. The source of hydrolyzed casein was Difco casamino acids. This was dissolved in distilled water at a concentration of 10 per cent prior to preparation of the medium, adjusted to pH 3.5 with hydrochloric acid, and treated with 10 per cent by weight of Darco G-60. After 15 minutes the Darco was filtered off and the clear solution used in the subsequent preparation of the medium.

The inoculum used in this assay procedure was found to be of great importance. It insured a low blank and a steep response curve upon the addition of graded amounts of vitamin B_c. It was prepared as follows:

Two stock cultures were prepared each month. One of these was maintained undisturbed, while subcultures were made from the other, weekly, in solid medium. 24 hours prior to the time an assay was to be set up a transfer was made from the subculture to a tube of liquid medium containing 5 cc. of the riboflavin medium of Snell and Strong (11), 5 cc. of water, and 1 γ of riboflavin. At the time of the inoculation, 1 cc. of this culture was diluted aseptically with sterile saline to 10 cc. and 1 drop of this dilution used for inoculum. The cultures were grown in Evelyn colorimetric tubes and their turbidity determined with this instrument after 40 hours incubation at 37°. This made it convenient to set up and inoculate the assay tubes in the afternoon and read them the morning of the 2nd day later.

Desiccated hog kidney proved to be a practical source of the enzyme. It was highly active, had a vitamin B_c content, or blank, low enough for most purposes, and was extremely stable. Desiccated hog intestine had a lower blank but this advantage was outweighed by the fact that it was more difficult to prepare in quantity. We have kept a sample of the desiccated kidney at room temperature over 2 years and can detect no change in its activity. The desiccated kidney used in these experiments (Table II, Method 1) was prepared by grinding fresh hog kidney and immersing it in 5 volumes of acetone. Following filtration, rewashing with acetone, and air drying, the residue was milled to a fine powder. An amount of sample expected to contain 25 to 50 γ of vitamin B_c was transferred to a small beaker. A few cc. of water and 0.3 gm. of powdered desiccated kidney were added. The mixture was adjusted to pH 4.3 to 4.5 by the addition of dilute HCl or NaOH. It was washed into a test-tube or Erlenmeyer flask; the volume was kept between 10 and 20 cc. A few drops of toluene were added and the sample incubated 48 hours at 37°. Then it was heated briefly in an autoclave, adjusted to pH 7.0, and diluted to 50 cc. After mixing and centrifuging, an aliquot of the supernatant was removed for microbiological assay.

The second enzyme preparation studied was an extract of almond (Method 2). This was prepared by grinding unheated almonds in a mortar while gradually adding either water or 0.1 M phosphate buffer of pH 7.0 until a total of 10 cc. had been added for each gm. of almond. The resulting emulsion was filtered through muslin. In making the enzyme preparation from almond meal (Method 3), an extract was made by adding water or phosphate buffer to commercial almond meal in the proportion of 3 cc. per gm. of meal. After occasional shaking of this mixture for half an hour it was filtered by suction through Super-Cel. An amount of sample containing approximately 25 to 50 γ of vitamin B_c was incubated with 10 cc. of either of the almond extracts described above for 48 hours at 37°. Following this it was treated exactly as described for samples treated with desiccated kidney.

The enzyme preparation used in Method 4 was made by grinding fresh hog kidney, adding 3 volumes of distilled water, and allowing the mixture to autolyze under toluene at 37° for about 36 hours. The insoluble material was centrifuged off and the supernatant clarified by filtering through a layer of diatomaceous earth (Super-Cel). An amount of this solution equivalent to about 0.8 gm. of fresh kidney was added to an amount of the substrate calculated to contain 25 to 50 γ of vitamin B_c in a test-tube. 5 cc. of 0.1 M acetate buffer of pH 4.5 and enough water to make 10 cc. were added, along with a few drops of toluene. This mixture was incubated 16 hours at 45°, heated, centrifuged, and assayed.

TABLE II

Comparison of Vitamin B_c Potencies Obtained by Chick Assay with Lactobacillus casei Assay Values before and after Enzyme Treatment

Substance No.	Substance assayed	Enzyme method No.	Vitamin B_c per gm.		
			Microbiological assay		Chick assay
			Initial	After enzyme	
			γ	γ	γ
96372	Yeast extract*	1	2.5	50	55
0742	Concentrate from yeast	1	11.6	198	200
38843	Yeast extract*	2	2.0	52	50
38843	" "	3	2.0	57	50
42773	Bacto-yeast extract†	1	0.7	26	25
45903	Concentrate from yeast	2	12.6	250	280
58264	Liver extract	1	20.0	26	54
58264	" "	2	20.0	52	54
65614	Concentrate from liver extract	2	34.0	89	98
71124	Asparagus juice concentrate‡	4	5.0	7	12
79914	Concentrate from yeast	4	48.0	7670	8135

Method 1, desiccated hog kidney, pH 4.3 to 4.5; Method 2, extract of almond, pH 6.0 to 7.0. Later time-reaction experiments with enzyme preparations from almond, on a purified substrate, have shown the optimum pH of this enzyme to be about pH 4.5; Method 3, extract of almond meal, pH 6.0 to 7.0; Method 4, extract of autolyzed hog kidney, pH 4.5.

* An aqueous extract of plasmolyzed yeast.

† Difco Laboratories, Detroit.

‡ Kindly supplied by Dr. Howard D. Lightbody, of the Western Regional Research Laboratory, Albany, California.

Recently we have modified the enzymatic digestion procedure somewhat in order to reduce the amount of substrate, and thereby increase the ratio of enzyme to substrate. This made it possible to obtain consistent results even with some samples which were inhibitory toward the enzyme. To

prepare the enzyme fresh hog kidney was blended and mixed with 3 parts of distilled water. This suspension was centrifuged and the centrifugate filtered through Super-Cel. The clarified extract was either dried from the frozen state and stored in the refrigerator, or dispensed in 5 cc. amounts in test-tubes and maintained in the frozen state in a dry ice chest.

The sample to be digested was diluted with water and an amount estimated to contain 1 to 2 γ of vitamin B_c in 1 or 2 cc. of water added to a test-tube marked at 10 cc. 5 cc. of 0.1 M acetate buffer of pH 4.5 and an amount of enzyme preparation equivalent to approximately 0.4 gm. of fresh hog kidney were added. The volume was then about 8 cc. A few drops of toluene were added and the mixture incubated 16 hours at 45°. The contents of the tube were then adjusted to pH 7.0 by the addition of N NaOH, the volume made up to 10 cc., and autoclaved about 5 minutes. Appropriate dilutions were then made for assay.

The biological assay values presented in Table II were obtained on chicks by the method recently published from this laboratory (12). The chick antianemia potencies were evaluated by comparison with a substandard, included in each assay, which has been assayed directly against crystalline vitamin B_c.

DISCUSSION

The microbiological assay procedure described above has not been found well suited for the determination of "free" vitamin B_c in natural substances whose major content of this vitamin is in the conjugated form. It is only after thorough treatment with a good source of vitamin B_c conjugase that such natural substances give response curves similar to the response curve for pure vitamin B_c which is used as the standard.

It will be noticed that the correlation between antianemia potency in chicks and microbiological assay values after enzyme treatment is quite good except in the case of asparagus juice concentrate and liver extract, when desiccated kidney is used as the enzyme. However, when almond was used as a source of enzyme, the microbiological value for liver extract was practically identical with the biological potency. This has not been true with all liver extracts, nor was it true with asparagus juice concentrate. Regardless of the enzyme preparation used with various plant extracts, we have been unable to obtain microbiological assays equal to antianemia potencies in chicks. This discrepancy may be due to the presence in plant extracts of inhibitors for the specific enzyme, or part of the chick activity may be due to the presence of one or more compounds which, even after treatment with the specific enzyme, are inactive for *Lactobacillus casei*.

Mallory *et al.* (6) have pointed out a correlation between microbiological assay values obtained for certain materials with *Streptococcus lactis*, follow-

ing enzyme treatment, and the corrective effect of these materials for the toxic symptoms produced in rats by succinylsulfathiazole. They have proposed that this effect on succinylsulfathiazole-treated rats be made the basis of a quantitative biological assay for vitamin B_c or related factors. We feel that a comparison of microbiological assay data, obtained after enzyme treatment, with antianemia potencies determined on chicks is of even more fundamental significance, since the anemia in chicks is a natural deficiency disorder and not an artificial condition imposed by a drug.

SUMMARY

Methods are described for the enzymatic liberation of vitamin B_c from the bound form in which it is often found in natural materials, making it possible to obtain microbiological assays with *Lactobacillus casei* representing what appeared to be the total amount of the vitamin present. Sources of the enzyme used were desiccated hog kidney, extract of fresh and autolyzed hog kidney, and extracts of whole almond or almond meal.

Microbiological assays of natural materials and concentrates with *Lactobacillus casei*, following these enzyme treatments, were for the most part in agreement with chick antianemia assays. There was an exception in the case of plant extracts, where no enzymatic procedure was found that would insure microbiological assay values equal to those obtained on chicks.

BIBLIOGRAPHY

1. Pfiffner, J. J., Binkley, S. B., Bloom, E. S., Brown, R. A., Bird, O. D., Emmett, A. D., Hogan, A. G., and O'Dell, B. L., *Science*, **97**, 404 (1943).
2. Binkley, S. B., Bird, O. D., Bloom, E. S., Brown, R. A., Calkins, D. G., Campbell, C. J., Emmett, A. D., and Pfiffner, J. J., *Science*, **100**, 36 (1944).
3. Welch, A. D., and Wright, L. D., *Science*, **100**, 153 (1944).
4. Totter, J. R., Mims, V., and Day, P. L., *Science*, **100**, 223 (1944).
5. Mims, V., Totter, J. R., and Day, P. L., *J. Biol. Chem.*, **155**, 401 (1944).
6. Mallory, M. E., Mims, V., Totter, J. R., and Day, P. L., *J. Biol. Chem.*, **156**, 317 (1944).
7. Laskowski, M., Mims, V., and Day, P. L., *J. Biol. Chem.*, **157**, 731 (1945).
8. Cheldelin, V. H., Eppright, M. A., Snell, E. E., and Guirard, B. M., *Univ. Texas Pub.*, *No. 4237*, 15 (1942).
9. Bird, O. D., Binkley, S. B., Bloom, E. S., Emmett, A. D., and Pfiffner, J. J., *J. Biol. Chem.*, **157**, 413 (1945).
10. Mitchell, H. K., and Snell, E. E., *Univ. Texas Pub.*, *No. 4137*, 36 (1941).
11. Snell, E. E., and Strong, F. M., *Ind. and Eng. Chem., Anal. Ed.*, **11**, 346 (1939).
12. Campbell, C. J., Brown, R. A., and Emmett, A. D., *J. Biol. Chem.*, **152**, 483 (1944).

THE PATHWAY OF DECOMPOSITION OF MYOADENYLIC ACID DURING AUTOLYSIS IN VARIOUS TISSUES

BY STANLEY E. KERR AND KRIKOR SERAIDARIAN

(From the Department of Biochemistry, American University of Beirut, Beirut, Lebanon)

(Received for publication, April 23, 1945)

In a previous study on the postmortem autolysis of adenosine triphosphate in brain (1) it was found that after the loss of two phosphate groups the resulting myoadenylic acid was further decomposed to nucleoside or free purine without any increase in inosinic acid. Adenine, hypoxanthine, and pentose were found in the fraction containing nucleosides and free purines, and as autolysis proceeded, hypoxanthine increased at the expense of adenine. Since enzymes for the deamination of adenine are known to be absent from the skeletal muscle of the rabbit, pig, and dog (2, 3), and from all human tissues (4), the progressive decrease of this purine suggests that it is deaminized in the form of riboside. The conclusion was therefore reached that in nerve tissue myoadenylic acid is dephosphorylated with the production of adenosine, rather than deaminized to form inosinic acid with subsequent dephosphorylation. This course of decomposition differs from that previously deduced for the muscles of the frog (5) and rabbit (6), but is similar to that suggested for embryonic tissue, liver, and Jensen's sarcoma of rats (7).

Our conclusions regarding the formation of adenosine in nerve tissue were based on the indirect evidence mentioned above, no methods being then available for the quantitative separation of nucleoside from free purine. The formation of adenosine, either in the normal catabolism of tissues or after injury, is of considerable interest from the pharmacological point of view (8), and a more direct approach to the problem is desirable. With this in view we developed methods for the quantitative separation of purine nucleosides from the free purine bases, and for the determination of the purines and ribose in these fractions (9). With these methods the course of autolysis of myoadenylic acid has been reinvestigated in the brain, skeletal and heart muscle, liver, and testes of the dog.

EXPERIMENTAL

The tissues were taken from dogs under amytal anesthesia, and immediately crushed in a mortar or ground in a meat chopper, previous experience having shown that the rate of catabolism of nucleotide is much greater in crushed than in intact tissue (1). The ground tissue was allowed to autolyze at 37° for specified periods, then transferred to 10 per cent trichloro-

acetic acid. Further details of the methods for handling fresh and autolysed tissues and the preparation of the protein-free filtrates are given elsewhere (1, 10). From these filtrates nucleotides were precipitated by uranium acetate, free purines by silver nitrate in acid solution, and nucleosides by silver nitrate in alkaline solution. The individual purines contained in each of these fractions, as well as ribose in the two silver precipitates, were then determined. The entire procedure, designed especially for the present study, is described in a recent paper (9). Organic phosphorus (*i.e.*, the

TABLE I

Distribution of Adenine, Hypoxanthine, and Guanine As Nucleotide (Uranium Precipitate), Nucleoside (Alkaline Silver Precipitate), and Free Purine (Acid Silver Precipitate) in Trichloroacetic Acid Extracts of Crushed Brain (Dog) after Various Periods of Autolysis

The results are expressed in millimoles per kilo of tissue.

Experiment No.	Period of autolysis	Nucleotide			Nucleoside						Free purine				
		Adenine	Hypoxanthine	Guanine	Adenine	Hypoxanthine	Guanine	Sum of purines	Ribose	Organic P	Adenine	Hypoxanthine	Guanine	Ribose	Organic P
	min.														
30-69	3	1.91	0.14	0.39*	0.57	0.10	0.05	0.72	0.87	0.27	0.0	0.11	0.03	0.11	0.09
30-70	10	1.21	0.13	0.32*	0.74	0.23	0.05	1.02	1.28	0.05	0	0.23	0.06	0.17	0.12
30-70	20	1.08	0.08	0.31*	0.69	0.35	0.04	1.08	1.23	0.09	0	0.42	0.07	0.16	0.12
29-38	1				0.18	0.09†		0.27	0.36	0	0.06	0.16†		0.27	0.18
29-39	5				0.47	0.16†		0.63	0.70	0.07	0	0.17†		0.11	0.07
29-40	10				0.29	0.31†		0.60	0.73	0	0	0.31†		0.19	0.18
29-41	15				0.58	0.27†		0.80	1.07	0.04	0.19	0.34†		0.35	0.36
30-44	20				0.29	0.73†		1.02	1.26	0	0.07	0.59†		0.15	0.15
29-42	30				0.33	0.42†		0.75	0.92	0.06	0.06	0.40†		0.25	0.11
29-43	60				0.13	0.59†		0.72	0.98	0.03	0.27	0.73†		0.19	0.20

* Double precipitation of purines as copper bisulfite compound.
† Includes guanine.

difference between total and inorganic phosphorus) was also determined (11) in the nucleoside and the free purine extracts, as a check on possible contamination with any nucleotide which might escape precipitation by uranium.

Results

A study of the results of analyses presented in Tables I to IV reveals dissimilar changes in the nucleotide metabolism in the different tissues of the dog during autolysis. As the adenylic acid disappears, no accumulation of

TABLE II

Distribution of Purines in Nucleotide (Uranium Precipitate), Nucleoside (Alkaline Silver Precipitate), and Free Purine (Acid Silver Precipitate) Fractions of Trichloroacetic Acid Extracts of Skeletal Muscle after Various Periods of Autolysis

The results are expressed as millimoles per kilo of tissue.

Experiment No.	Kind of muscle; treatment	Period of autolysis	Nucleotide			Nucleoside*			Free purine*		
			Adenine	Hypoxanthine	Guanine	Hypoxanthine and guanine	Ribose	Organic P	Hypoxanthine and guanine	Ribose	Organic P
		min.									
32-1	Dog, frozen in liquid air	0	4.20	−0.02	0.14†	0.02	0.09		0.03‡	0.08	0
30-64	Dog, minced	10	1.94	0.88	0.24						
30-64	" "	20	1.01	0.26	0.12						
30-64	" "	30	0.83	0.16	0.12						
30-64	" "	60	0.54	0.25	0.09						
30-45	" "	10				0.88	1.09	0.06	0.35	0.35	0.17
30-47	" "	20				1.54	1.85	0	0.69	0.37	0
30-46	" "	30				1.64	2.04	0.09	0.85	0.36	0.18
30-48	" "	60				2.36	2.76	0	2.03	0.42	0.05
32-2	" auto-lyzed intact	20	5.00	0.02	0.15†						
32-4	Beef, auto-lyzed intact	300	4.90	0.92	0.18	0.58	1.15	0.18	0.25	0.41	0.18

* No adenine was found in this fraction in any experiment of this group.

† Double precipitation of purines as copper bisulfite compound.

‡ Includes 0.007 mM of guanine.

TABLE III

Distribution of Adenine, Hypoxanthine, and Guanine As Nucleotide (Uranium Precipitate), Nucleoside (Alkaline Silver Precipitate), and Free Purine (Acid Silver Precipitate) in Trichloroacetic Acid Extracts of Minced Heart Muscle of the Dog, after Various Periods of Autolysis

The results are expressed in millimoles per kilo of tissue.

Experiment No.	Period of autolysis	Nucleotide			Nucleoside						Free purine*			
		Adenine	Hypoxanthine	Guanine	Adenine	Hypoxanthine	Guanine	Sum of purines	Ribose	Organic P	Hypoxanthine	Guanine	Ribose	Organic P
	min.													
30-75	0	6.17	−0.04	0.21†	0.07	0.07	0.02	0.20	0.03	0.17	0.07	0.06	0	0.03
30-72	3.5	4.24	−0.03	0.22†	0.81	0.39	0.04	1.24	1.83	0	0.12	0.05	0	0
30-72	10	2.07	0.02	0.16†	1.57	2.15	0.15	3.87	4.13	0.07	0.52	0.08	0.33	0.07

* No adenine was found in this fraction in any experiment of this group.

† Double precipitation of purines as copper bisulfite compound.

TABLE IV

Distribution of Adenine, Hypoxanthine, and Guanine As Nucleotide (Uranium Precipitate), Nucleoside (Alkaline Silver Precipitate), and Free Purine (Acid Silver Precipitate) in Trichloroacetic Acid Extracts of Minced Liver (Dog) and Testes (Dog and Ox)

The results are expressed in millimoles per kilo of tissue.

Experiment No.	Tissue	Period of autolysis	Nucleotide			Nucleoside						Free purine				
			Adenine	Hypoxanthine	Guanine	Adenine	Hypoxanthine	Guanine	Sum of purines	Ribose	Organic P	Adenine	Hypoxanthine	Guanine	Ribose	Organic P
		min.														
30-76	Dog liver	0	3.86	−0.04	0.39*	0.26	0.03	0.04	0.33	0.06	0.06	0	0.02	0.10	0	0
30-73	" "	2.5	3.26	0.31	0.22*	0.12	0.26	0.16	0.54	1.06	0.02	0	0.06	0.04	0	0
30-67	" "	10	1.84	0.28	0.32	0.51	0.44	0.10	1.05	1.29	0.06	0.16	0.31	0.10	0.26	0.54
30-67	" "	30	0.25	0.56	0.12	0.10	0.71	0.13	0.94	0.95	0.06	0.51	1.67	0.25	0.25	0.95
30-67	" "	60	0.25	0.28	0.14	0.13	1.88	0.39	2.40	0.81	0.22	0.11	1.19	0.17	0.22	0.18
30-74	" testes	0	4.13	−0.01	0.33*	0	0.06	0.02	0.08	0	0.13	0	0.04	0.02	0	0.03
30-71	" "	2	3.11	−0.10	0.41*	0.13	0.17	0.04	0.24	0.95	0	0	0.02	0.02	0	0
30-68	Ox "	10	1.07	−0.13	0.60	0.39	0.69	0.12	1.20	1.43	0	0	0.50	0.18	0.23	0
30-68	" "	30	0.91	−0.02	0.53	0.06	0.68	0.08	0.82	1.08	0	0	0.94	0.22	0.25	0
30-68	" "	60	1.04	0.09	0.47	0.06	0.55	0.08	0.69	0.89	0	0	0.98	0.20	0.24	0

* Double Precipitation of purines as copper bisulfite compound.

its deamination product inosinic acid occurs in brain, heart muscle, or testes, while in liver and skeletal muscle the increase of inosinic acid represents only a small fraction of the original nucleotide.

In the *nucleoside fraction* adenine is found in significant quantities in brain, heart muscle, liver, and testes, but not in skeletal muscle. Hypoxanthine is found as nucleoside in all of the tissues, but in the largest quantity in skeletal muscle. Ribose in the nucleoside fraction is in general somewhat higher than the sum of the purines. Organic phosphorus in this fraction is either absent or small in amount compared to the ribose and purine content, thus excluding significant contamination with nucleotide.

In the *free purine fraction* only traces of adenine are found in brain and liver, but none in testes and skeletal and heart muscle, the chief constituent of the free purine fraction being hypoxanthine in each of the tissues examined. Some pentose is found in the acid silver precipitate in most of the specimens.

The adenine nucleotide content of the several tissues studied varies between 3 and 6 mM per kilo of fresh tissue, heart muscle being the richest, skeletal muscle and liver containing about a third less, and brain about half as much.

Guanine nucleotide varies between a minimum concentration of 0.14 mM per kilo in skeletal muscle to 0.39 mM in brain and liver, with heart muscle intermediate. Guanine nucleotide represents 9 to 13 per cent of the total acid-soluble nucleotide in brain, liver, and testes, but only 3 per cent in skeletal and heart muscle. During autolysis the decomposition of this nucleotide is not as extensive as with adenylic acid. Guanine in the nucleoside fraction increases significantly in heart and liver.[1]

For the sake of comparison of autolysis in intact and minced muscle, a specimen was removed from a dog 20 minutes after death, then ground and immediately fixed in trichloroacetic acid. The results (Table II) show the adenine and guanine nucleotides at the resting level, and practically no inosinic acid present. In a similar experiment with beef muscle autolysis

[1] Hitchings (12) recommended double precipitation of the purines as copper bisulfite complex before performing the colorimetric determination of guanine, in order to eliminate extraneous chromogenic substances. This we did with most of the nucleotide fractions, as recorded in Tables I to IV, but not with the nucleoside and free purines in the silver precipitates, because of danger of loss in handling the very small amounts of purine. The amount of interfering chromogenic substance in the silver precipitates should be much less than in digests of whole tissue, which Hitchings used for the determination of total guanine, but the values presented in our tables may be high, especially after extensive autolysis, for the increase observed in guanosine and free guanine is greater than the decrease in guanine nucleotide. The negative values reported for hypoxanthine in some experiments are explained in our recent publication (9) as being due to the precipitation of small amounts of guanine with adenine picrate.

during 5 hours after death caused the loss of only a quarter of the adenylic acid, 14 per cent forming inosinic acid and 12 per cent nucleoside or free purine, changes which correspond to those taking place within about 10 minutes in minced dog muscle.

DISCUSSION

Indirect evidence presented in an earlier publication (1) and reviewed in the introduction above indicated that the pathway of catabolism of adenosine triphosphate in autolyzing brain is by way of adenosine. This is now confirmed by separating adenine together with an equivalent amount of pentose from the extract of crushed brain by methods especially designed to precipitate nucleosides (9).

Our results show that the same pathway of decomposition of adenine nucleotide, *i.e.*, by way of adenosine, is also followed in minced heart muscle, testes, and liver of the dog, but not in skeletal muscle.

Although Parnas (5) and Ostern (6) had excluded adenosine and adenine as by-products in the catabolism of adenylic acid in frog, rabbit, and beef skeletal muscle, Ostern and Mann (13) stated that in some experiments they had had evidence which led them to suspect the formation of adenosine in certain tissues. They finally concluded that the pathway of decomposition of adenylic acid is by way of dephosphorylation to adenosine, rather than by direct deamination, in those tissues in which adenosine is deaminized more rapidly than adenylic acid, *e.g.*, heart muscle. Reis attempted to secure more direct evidence on the ability of these tissues to dephosphorylate nucleotides, and this led to the discovery of the specific 5-nucleotidase (14). The ability of various tissues or their extracts to remove phosphorus from the purine nucleotides had already been demonstrated by many workers (6, 15), but this action was attributed to the non-specific alkaline phosphatase (16).

Since enzymes capable of removing phosphorus from nucleotides were known to be present in various tissues, as noted above, and also deaminases capable of splitting the amino group from both adenylic acid and adenosine (2), a number of studies were made to determine whether deamination preceded the loss of phosphorus or *vice versa*. After a series of conflicting reports had appeared (17), Parnas, Ostern, and Mann (18) presented evidence that only adenylic acid, not adenosine triphosphate, is deaminized by muscle brei. Our results with autolyzing brain (1) support this conclusion, for no deamination of nucleotide occurs until two of the three phosphorus groups attached to the adenosine triphosphate have been lost (1 minute).

The further decomposition of adenylic acid appears to vary with different tissues and their treatment. According to von Euler and Skarzynski (7)

the dephosphorylating mechanism seems to predominate over that for deamination in liver, embryonic tissue, and Jensen's sarcoma of the rat. Our analyses confirm this for liver by the finding of adenosine.

Deamination of the nucleotide predominates over dephosphorylation in skeletal muscle. Parnas (5), on grinding frog muscle with water, found the adenylic acid converted only to inosinic acid with no loss of the acid-stabile phosphorus group. With rabbit muscle 1 to 6 hours post mortem Ostern (6) accounted for 40 to 50 per cent of the nucleotide as inosinic acid, and about 20 per cent as hypoxanthine in the nucleoside and free purine fractions. In dog muscle the maximum amount of inosinic acid we observed (10 minutes after mincing) represented only one-fifth of the original adenylic acid. With intact beef muscle even less than this was found 5 hours post mortem. Our failure to find either adenosine or free adenine in skeletal muscle confirms in part the work of Parnas and Ostern, but under the conditions of our experiments inosinic acid formation was much less than they observed.

Reasons for the divergence of the pathway of decomposition in various tissues must be sought in the enzyme systems governing the splitting of the nucleotide molecule at its vulnerable points. In a tissue containing deaminases, phosphatases, and possibly N-nucleotidase (19, 20), each capable of decomposing adenylic acid in a different way, the course of events will depend on the relative amounts of the various enzymes, and on conditions which affect the activity of each, such as acidity, the concentration of activators, and the formation of split-products (ammonia, inorganic phosphate, adenosine, or inosine) which may inhibit certain reactions. Since the content of these enzymes and of metallic ions such as magnesium and calcium varies widely in different tissues, the pathway of decomposition, being the resultant of the competition of the enzymes for the substrate, may also differ. Since the N-nucleotidase probably plays a minor rôle, the pathway of decomposition for adenylic acid is determined by the competition between the deaminizing and the dephosphorylating enzyme systems. Predominance of the latter results in the formation of adenosine, which is then subject to deamination, whereas if deamination proceeds at a more rapid rate than dephosphorylation the result is inosinic acid, which in turn loses phosphorus to become inosine.

It is of interest, therefore, to compare the phosphatase content of such tissues as skeletal muscle, in which adenosine is not formed, with brain or heart in which adenosine is readily produced during autolysis. Reis (14), studying the activity of a number of tissues, found the white substance of calf brain to be one of the richest sources of the specific 5-nucleotidase, whereas frog muscle contains a negligible amount, thus explaining the fact

that in frog muscle the adenylic acid is only deaminized to inosinic acid, with no loss of phosphate (5), while in brain dephosphorylation proceeds more rapidly than deamination and adenosine is produced, as shown in this paper.

Further work is necessary to determine whether the pathway of decomposition of adenylic acid is uniform in the same organ of different animals, for the findings of Reis (14) suggest a great variability in the nucleotidase content of heart muscle, rabbit heart being poor, rat and frog heart rich in this enzyme.

It should be noted that not only the 5-nucleotidase but also the nonspecific alkaline phosphatase of bone, intestine, etc., is a powerful agent in removing phosphorus from the nucleotides (21). Both enzymes may participate in the breakdown of nucleotide to nucleoside, the pH range for activity being the same for each.

Ishikawa and Komita (19) and Komita (20) demonstrated the existence of a glucosidase (N-nucleotidase) in dog pancreas, capable of splitting guanylic and xanthylic acids, but no studies were made on adenylic acid. Decomposition of adenylic acid by this enzyme would produce free adenine. In our studies the quantities of adenine found in the free purine fraction of brain and liver are so minute that we prefer to withhold any interpretation until these results can be checked with improved methods.[2]

The occurrence of pentose in the acid silver precipitate needs explanation. In our study on methods (9) it was found that, although nucleoside in pure solution is not precipitated in acidic medium, some of it may be carried down with silver chloride. Any nucleotide which escapes precipitation by uranium acetate should be precipitated later by silver nitrate in alkaline but not in acid solution (9), but as already stated the amount of organic phosphorus in the alkaline silver precipitate is negligible. In the acid silver precipitate there is some organic phosphorus, but it corresponds roughly to the pentose concentration only in brain filtrates; hence it is unlikely that the pentose found here represents either nucleotide or ribose phosphate.

The physiological significance of tissue deaminases and phosphatases with specific activity for adenylic acid is not yet understood. The powerful vaso-depressor properties possessed by adenosine but not by inosine (8) and the fact that in the catabolism of adenylic acid adenosine is produced in nerve tissue but not in skeletal muscle suggest the possibility that the liberation of adenosine from the nerve endings may play a rôle in the control of the capillary circulation.

[2] The presence of guanine makes the determination of small amounts of adenine unreliable (9). Studies are in progress to adapt the present methods to the determination of much smaller quantities of adenine without interference from guanine.

SUMMARY

1. The course of decomposition of adenylic acid has been studied during postmortem autolysis in minced brain, skeletal and heart muscle, liver, and testes of the dog, and in ox testes.

2. As adenylic acid disappears, no accumulation of inosinic acid occurs in brain, heart, or testes. In liver and skeletal muscle only a small proportion of the original nucleotide appears as inosinic acid.

3. Decomposition of adenylic acid takes place much more slowly in intact than in minced tissues.

4. Adenosine is produced in brain, heart muscle, liver, and testes, but not in skeletal muscle. Inosine is found in all the autolyzed tissues, but in the greatest amounts in skeletal muscle.

5. The chief constituent of the free purine group is hypoxanthine in each of the tissues studied.

6. Of the total acid-soluble nucleotide, 9 to 13 per cent is guanylic acid in brain, liver, and testes, and about 3 per cent in skeletal and heart muscle. Preliminary results indicate that guanosine is produced during autolysis in heart and liver.

BIBLIOGRAPHY

1. Kerr, S. E., *J. Biol. Chem.*, **145**, 647 (1942).
2. Schmidt, G., *Z. physiol. Chem.*, **208**, 185 (1932).
3. Leonard, V. N., and Jones, W., *J. Biol. Chem.*, **6**, 453 (1909).
4. Winternitz, M. G., and Jones, W., *Z. physiol. Chem.*, **60**, 180 (1909).
5. Parnas, J. K., *Biochem. Z.*, **206**, 16 (1929).
6. Ostern, P., *Biochem. Z.*, **221**, 64 (1930).
7. von Euler, H., and Skarzynski, B., *Z. physiol. Chem.*, **263**, 259 (1940).
8. Ringler, R., in Heffter, A., Handbuch der experimentellen Pharmakologie, Berlin, Ergänzungwerk, **7**, 63 (1938).
9. Kerr, S. E., and Seraidarian, K., *J. Biol. Chem.*, **159**, 211 (1945).
10. Kerr, S. E., *J. Biol. Chem.*, **110**, 625 (1935).
11. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
12. Hitchings, G. H., *J. Biol. Chem.*, **139**, 843 (1941).
13. Ostern, P., and Mann, T., *Biochem. Z.*, **260**, 326 (1933).
14. Reis, J., *Bull. Soc. chim. biol.*, **16**, 385 (1934); *Enzymologia*, **2**, 110, 183 (1937).
15. Levene, P. A., and Dillon, R. T., *J. Biol. Chem.*, **88**, 753 (1930). Mozolowski, W., *Biochem. Z.*, **206**, 150 (1929). Dmochowski, A., *Biochem. J.*, **23**, 1346 (1929). Jacobsen, E., *Biochem. Z.*, **242**, 292 (1931). Barrenscheen, H. K., and Lang, S., *Biochem. Z.*, **253**, 395 (1932).
16. Folley, S. J., and Kay, H. D., in Nord, F. F., and Weidenhagen, R., Ergebnisse der Enzymforschung, Leipzig, **5**, 159 (1936).
17. Barrenscheen, H. K., and Filz, W., *Biochem. Z.*, **250**, 281 (1932). Lohmann, K., *Biochem. Z.*, **254**, 381 (1932). Mozolowski, W., and Sobczuk, B., *Biochem. Z.*, **265**, 41 (1933).
18. Parnas, J. K., Ostern, P., and Mann, T., *Biochem. Z.*, **272**, 64 (1934).
19. Ishikawa, H., and Komita, Y., *J. Biochem.*, Japan, **23**, 351 (1936).
20. Komita, Y., *J. Biochem.*, Japan, **25**, 405 (1937); **27**, 23 (1938).
21. Schmidt, G., and Thannhauser, S. J., *J. Biol. Chem.*, **149**, 369 (1943).

COPPER AND ZINC IN EPIDERMAL CARCINOGENESIS INDUCED BY METHYLCHOLANTHRENE*

By CHRISTOPHER CARRUTHERS and V. SUNTZEFF

(From the Research Department of the Barnard Free Skin and Cancer Hospital, and the Department of Anatomy, Washington University School of Medicine, St. Louis)

(Received for publication, May 25, 1945)

The rôle of the alkalies, alkaline earths, and of iron in epidermal carcinogenesis in mice has been described (1) and the integration of the chemical, physical, and histological changes in mouse epidermis undergoing carcinogenesis has been recently reviewed in a second summarizing report by Cowdry (2). The experiments here recorded to determine the epidermal copper and zinc contents were made to complete this chapter of our studies.

EXPERIMENTAL

Since we have limited samples of tissue for analysis, microprocedures are essential. Therefore, a polarographic method was devised for the determination of traces of copper (3). With this procedure recoveries of small amounts of this metal could be accurately determined in standard solutions containing the cations which we previously had found in mouse epidermis. However, interference due to another metal was encountered when the procedure was applied to hydrochloric acid solutions of epidermal ash (3). The current-voltage curve which appeared on our polarograms was proved to be zinc, a fact which we had not anticipated. Since 0.1 N potassium thiocyanate is a supporting electrolyte for both copper and zinc (4) and since the half wave potentials of these metals are widely separated (4), it was impossible to determine both metals on the same tissue sample. Furthermore, ferric iron does not interfere as it does not produce a diffusion current in the potential range used in these studies. For calibration purposes a standard stock solution of zinc chloride was made by dissolving a weighed amount of reagent grade metallic zinc in dilute hydrochloric acid, and the copper content of a copper sulfate solution (approximately 0.06 N from cupric sulfate) was determined iodometrically by the procedure of Foote and Vance (5).

For analysis the sample of tissue was completely ashed at 450° in a silica crucible in a muffle furnace, and after cooling to room temperature the ash was dissolved in the crucible with 1 ml. of 0.1 N hydrochloric acid, to which were added 1 or 2 drops of concentrated nitric acid to insure complete

* This investigation was aided by grants from The International Cancer Research Foundation, the National Cancer Institute, and an anonymous donor.

oxidation of the ferrous to the ferric ion. This solution was evaporated to dryness on a steam bath and just before analysis the residue was dissolved in the crucible with 1 ml. of 0.1 N potassium thiocyanate. Then the entire contents of the crucible were poured into a small shell vial, the oxygen removed with a stream of nitrogen bubbles, and the copper and zinc contents determined polarographically (4). Nucleoprotein phosphorus was used as a basis of reference for the amount of tissue involved (1).

New Buffalo mice of both sexes were used and were painted with methylcholanthrene (0.6 per cent weight per volume in reagent grade benzene) as in our previous work (1). In this investigation all mice were sacrificed 5 days after the last treatment with the carcinogen instead of being killed from 1 to 4 days as was done heretofore, in order to make the interval between the last application and the time of sacrificing the animal the same in all our studies. All mice were maintained in glass jars until weaned, at which time they were placed in wooden boxes which were metal-free on the inside. This precaution was necessary because mice that had been reared in galvanized wire cages were found to contain more epidermal zinc than those raised in metal-free containers.

The transplantable carcinoma which we have used for all analyses has passed through twenty-three generations, and has remained practically the same morphologically. The tumor has been described by Cooper, Firminger, and Reller (6). The tumor transplants were removed about 10 days after implantation, at which time they were small, solid, and with little, if any, necrosis. A pool of many tumors weighing between 800 and 2000 mg. (wet weight) was employed for each analysis of copper and zinc.

DISCUSSION

The results of our investigations on the mineral composition of mouse epidermis undergoing carcinogenesis are shown graphically in Fig. 1. The time in days is plotted against the percentage of change in the metal-nucleoprotein phosphorus (NPP) ratios. The possible significance of the changes in the minerals, except for copper and zinc, in hyperplastic epidermis and in the carcinoma has been mentioned (1). From Fig. 1 it is apparent that three applications of methylcholanthrene lowered the Cu:NPP ratio at 10 days to 45 per cent of normal. This drop is maintained essentially the same for 20, 30, and 60 days, at which time the mice had received six, twelve, and twenty-four treatments, respectively, of the carcinogen. The copper content of the carcinoma is very low, since the Cu:NPP ratio was lowered to 83 per cent of normal. On the other hand the Zn:NPP ratio was diminished only to 20 per cent of normal after three treatments with the carcinogen, and at 20, 30, and 60 days the diminution of about 30 per cent was maintained. However, in the carcinoma the Zn:NPP ratio was 68 per cent of normal, a considerable decrease.

An over-all study of Fig. 1 reveals that magnesium, sodium, and potassium are not appreciably altered in hyperplastic epidermis, whereas calcium and iron are very low. Zinc and copper appear to occupy an intermediate position. These studies show that in hyperplastic epidermis a new chemical equilibrium is established as early as 10 days after one treatment with the carcinogen, and is maintained essentially unchanged

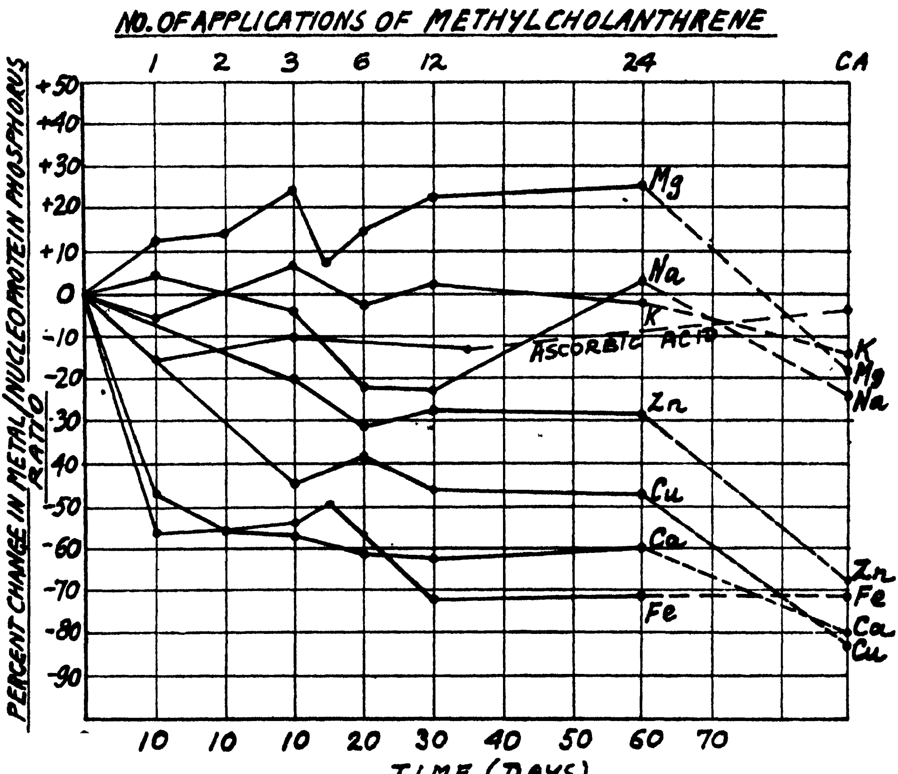


FIG. 1. Magnesium, sodium, potassium, calcium, iron, copper, zinc, and ascorbic acid in epidermal carcinogenesis induced by methylcholanthrene. *CA* represents carcinoma. The dotted lines (except for ascorbic acid from 35 days to 60 days) are used to designate the change from late hyperplastic epidermis to carcinoma.

until carcinoma develops. It will also be noted that the rate of change of the minerals up to 30 days appears to be in a state of flux which gives way to a stable state between 30 and 60 days.

In the transplantable carcinoma calcium and the heavy metals, zinc, iron, and copper, are very low. (The Fe:NPP ratio shown on the graph includes blood hemoglobin, and may be expected to show a further drop

when the latter is excluded.) Therefore, the carcinoma appears to have established still a different chemical equilibrium than that of hyperplastic epidermis as judged from the mineral composition. The two-step decreases in the calcium, iron, copper, and zinc contents reveal that the process of epidermal carcinogenesis occurs in two distinct phases: from normal to hyperplastic epidermis and from the latter to the cancer cell.

The physiological importance of zinc in the animal organism was established for the first time by Keilin and Mann's discovery that the enzyme carbonic anhydrase is a zinc-containing protein (7). Zinc has also been found in purified preparations of uricase (8, 9), and the rôle of this metal in the storage of insulin has been considered (10–12). However, the importance, if any, of zinc and of copper in carcinogenesis in mice must await further investigation.

TABLE I

Copper and Zinc Content in Epidermal Methylcholanthrene Carcinogenesis

Tissue	No. of mice	No. of analyses	Cu per 100 mg. tissue	Zn per 100 mg. tissue
			γ	γ
Normal epidermis	69	5	0.58	5.2
Benzene-treated epidermis	57	5	0.58	5.5
Methylcholanthrene-treated epidermis	115	14	0.33	3.8
Carcinoma	92	9	0.10	1.7

The amounts of copper and zinc in normal, benzene-treated, and in hyperplastic epidermis and in the carcinoma are shown in Table I.

SUMMARY

The rôle of copper and zinc in epidermal carcinogenesis in mice induced by methylcholanthrene is discussed. Hyperplastic epidermis was found to contain about 45 per cent less copper and about 30 per cent less zinc than does normal epidermis. The transplantable squamous cell carcinoma was very low in both copper and zinc, having 83 per cent less of the former and 68 per cent less of the latter than that of untreated epidermis. The possible signification of the rôle of the minerals in the process of transformation of normal epidermis to carcinoma is briefly described, and the evidence to date indicates that the transformation of normal mouse epidermis to squamous cell carcinoma occurs in two distinct phases.

BIBLIOGRAPHY

1. Carruthers, C., and Suntzeff, V., *J. Biol. Chem.*, **155**, 459 (1944).
2. Cowdry, E. V., *J. Invest. Dermat.*, **6**, 15 (1945).

3. Carruthers, C., *Ind. and Eng. Chem., Anal. Ed.*, in press.
4. Kolthoff, I. M., and Lingane, J. J., Polarographic analysis and voltammetry amperometric titrations, New York (1941).
5. Foote, H. W., and Vance, J. E., *Ind. and Eng. Chem., Anal. Ed.*, **8**, 119 (1937).
6. Cooper, Z. K., Firminger, H. I., and Reller, H. C., *Cancer Res.*, **4**, 617 (1944).
7. Keilin, D., and Mann, T., *Nature*, **144**, 442 (1939); *Biochem. J.*, **34**, 1163 (1940).
8. Davidson, J. N., *Biochem. J.*, **32**, 1386 (1938).
9. Holmberg, C. G., *Biochem. J.*, **33**, 1901 (1939).
10. Scott, D. A., and Fischer, A. M., *J. Clin. Invest.*, **17**, 725 (1938).
11. Fischer, A. M., and Scott, D. A., *Biochem. J.*, **29**, 1055 (1935).
12. Eisenbrand, J., and Sienz, M., *Z. physiol. Chem.*, **268**, 1 (1941).

INVESTIGATIONS OF AMINO ACIDS, PEPTIDES, AND PROTEINS

XXI. THE DETERMINATION OF HISTIDINE IN PROTEIN HYDROLYSATES WITH LEUCONOSTOC MESENTEROIDES P-60*

By MAX S. DUNN, MERRILL N. CAMIEN, S. SHANKMAN, AND LOUIS B. ROCKLAND

(*From the Chemical Laboratory, University of California, Los Angeles*)

(Received for publication, April 3, 1945)

Reasonably satisfactory microbiological procedures (2) for the determination in proteins of arginine, aspartic acid (3), isoleucine, glutamic acid, leucine, lysine (4), serine (3), tryptophane, and valine with lactobacilli have been described previously but a microbiological method for the determination of histidine has not been reported. A procedure for the determination of this amino acid with *Leuconostoc mesenteroides* P-60 is described in this paper.

EXPERIMENTAL

Solutions of the standard amino acid, the unknown, and the basal medium were transferred to the tubes with the aid of a Brewer automatic pipette (Baltimore Biological Laboratories). It was found that 1 ml. volumes of solution are delivered by this instrument with an error not greater than 0.1 per cent. Solutions may be pipetted with this apparatus more conveniently, with greater accuracy, and in much less time than by hand.

It has been shown in experiments not reported in this paper that the growth-promoting activity of *dl*-histidine was only one-half that of *l* (−)-histidine.

It seemed desirable to determine what, if any, modifications in the composition of basal Medium D given in Table I of a previous publication (5) were required so that the capacity of the medium to resist the stimulatory or inhibitory influence of amino acids or other substances introduced in an assay of histidine would be approximately equivalent to that found previously to be satisfactory in the assay of lysine. A series of experiments on multiple media entirely analogous to those described previously in a comparable investigation of lysine (5) was performed but the curves showing the response of the microorganism to different concentrations of histidine at varying levels of components of the medium have been omitted in order that space might be conserved.

* For Paper XX in this series see Dunn *et al.* (1). This work was aided by grants from the Gelatin Products Company, Merck and Company, Inc., the Nutrition Foundation, Inc., Schering and Glatz, and the University of California.

It was found from measurements over a range of concentration of $l(-)$-histidine up to 25 γ per tube that acid production was nearly constant for the stipulated constituents over the ranges of concentrations shown in the parentheses: total amino acids (1.5- to 2.5-fold levels), glucose (2 to 4 per cent), sodium acetate (1.2 to 2.4 per cent), purines and pyrimidines (adenine, guanine, and uracil with and without xanthine, 1- to 5-fold levels), buffer and other salts except sodium acetate (0.5- to 5-fold levels), and vitamins (0.3- to 2-fold levels). Acid production decreased markedly at all levels of histidine up to 0.3 per cent sodium chloride but it decreased gradually beyond this and up to 2.5 per cent sodium chloride. There was a continuous increase in acid production up to 1.2 per cent concentration of ammonium chloride. Growth (*i.e.*, turbidities measured with a photoelectric colorimeter (Lumetron)) was constant at each level of histidine up to 1.2 per cent concentration of ammonium chloride. It would appear, therefore, that assays of histidine in unknown solutions containing relatively large amounts of this salt might be made satisfactorily by a turbidimetric procedure. It would seem desirable in such instances, however, to investigate more completely the influence of other constituents of the medium on the growth of the microorganism.

In view of the statement of McMahan and Snell (6) that *Leuconostoc mesenteroides* and other microorganisms have a temperature optimum near 30°, growth and acid production were measured at 28° and 35° at five levels of $l(-)$-histidine up to 6.4 γ per tube and at intervals of 24, 48, 68, and 117 hours. Smooth standard curves were obtained in all cases and total acid production at the highest level of histidine was equivalent to 1.5, 5.5, 7.5, and 10 ml. of 0.02 N sodium hydroxide at 28° and to 5, 8, 11, and 13 ml. at 35°.

On the basis of the described experiments, it was concluded that the basal medium and the experimental conditions employed previously for the assay of lysine would probably be equally satisfactory for the assay of histidine. Although it was demonstrated that no synthesis of histidine occurred even over a 6 day period, it was found that the standard curves were most satisfactory when the microorganisms grew for 5 days. The casein, the silk fibroin, and the hydrolysis procedure were the same as those described earlier (2) and the assay technique was the same as that given in previous publications.

It was determined in an experiment designed to test the precision of the titration values (Table I) that there was relatively little variation in the number of ml. of 0.05 N sodium hydroxide required to titrate the acid produced in thirteen replicate samples at each of ten levels of $l(-)$-histidine and that the mean deviation from the mean for the entire 130 titrations was only 1.8 per cent. It appeared, therefore, that the titration values did not vary significantly at different positions in the rack or the incubator and that

TABLE I

*Reproducibility of Titration Values with Leuconostoc mesenteroides P-60 at Ten Levels of l(−)-Histidine**

Histidine per 5 ml.	Titration volume of 0.05 N NaOH	
	Range	Mean
γ	*ml.*	*ml.*
0	1.18- 1.32	1.24 ± 0.04
2	2.45- 2.83	2.62 ± 0.08
4	3.76- 4.03	3.87 ± 0.05
6	4.85- 5.12	5.01 ± 0.06
8	5.88- 6.21	6.02 ± 0.10
10	6.77- 7.49	6.95 ± 0.16
12.5	7.81- 8.51	8.02 ± 0.12
15	8.90- 9.28	9.09 ± 0.12
20	10.30-11.15	10.69 ± 0.17
25	11.70-12.12	11.92 ± 0.12

* 3 day incubation period. The solutions were pipetted by hand. There were thirteen replicate samples at each level of histidine. The total final volume of solution was 5 ml. per tube.

TABLE II

*Results of Assay with Leuconostoc mesenteroides P-60 of Histidine in Casein Hydrolysate**

Casein per tube	Titration volume of 0.046 N NaOH per tube	Histidine found	
		Per tube	Per ml. sample
γ	*ml.*	γ	γ
746	2.82	2.15	2.15
746	2.90	2.24	2.24
1492	4.63	4.45	2.22
1492	4.78	4.65	2.32
2238	6.10	6.45	2.15
2238	6.17	6.53	2.18
2984	7.93	8.92	2.23
2984	7.58	8.50	2.12
3730	9.20	10.65	2.15
3730	9.10	10.50	2.12

* An average of 2.19 γ of *l*(−)-histidine was found per ml. of casein hydrolysate. The percentage of histidine in the casein uncorrected for moisture and ash was calculated to be 2.93 per cent. It was found from sixteen comparable assays of the same casein hydrolysate that the histidine in casein uncorrected for moisture and ash ranged from 2.75 to 3.06 per cent and averaged 2.91 per cent. The average mean deviation from the mean at the different levels was 2.3 per cent in all assays.

In a series of five experiments, the percentages of added histidine recovered from casein hydrolysates ranged from 90 to 99 per cent and averaged 95.8 per cent.

the standard curves were satisfactorily reproducible. The precision and accuracy of the histidine assay data were determined by a series of experi-

TABLE III

*Results of Assay with Leuconostoc mesenteroides P-60 of Histidine in Amino Acid Test Mixture 1**

Amino acid mixture per tube	Titration volume of 0.046 N NaOH per tube	Histidine found	
		Per tube	Per ml. sample
γ	*ml.*	γ	γ
575	2.50	1.05	1.05
575	2.52	1.07	1.07
1150	3.64	2.15	1.08
1150	3.59	2.10	1.05
1725	4.62	3.10	1.03
1725	4.67	3.15	1.05
2300	5.66	4.08	1.02
2300	5.58	4.00	1.00
2875	6.52	4.90	0.98
2875	6.68	5.05	1.01

* The composition of the test mixture simulating casein was the same as that given in a previous paper (7) except that the mixture contained 1.74 per cent of $l(-)$-histidine. An average of 1.03 γ of $l(-)$-histidine was found per ml. of the test solution. The percentage of histidine in the amino acid test mixture was calculated to be 1.80 per cent. The percentage of histidine recovered was 103.2 per cent. In a series of twelve analogous experiments, the average mean deviation from the mean of the values at the different levels was 2.2 per cent and the recoveries of histidine ranging from 97.5 to 106.2 per cent averaged 102.9 per cent.

TABLE IV

*Results of Assay with Leuconostoc mesenteroides P-60 of Histidine in Amino Acid Test Mixture 2**

Amino acid mixture per tube	Titration volume of 0.046 N NaOH per tube	Histidine found	
		Per tube	Per ml. sample
γ	*ml.*	γ	γ
240	2.71	2.01	2.01
240	2.79	2.10	2.10
480	4.20	3.90	1.95
480	4.19	3.91	1.96
720	5.58	5.74	1.91
720	5.62	5.76	1.92
960	7.07	7.80	1.92
960	7.01	7.70	1.95
1200	8.39	9.50	1.90
1200	8.31	9.40	1.88

* Contained 100 mg. per 100 ml. of each of the amino acids listed in Test Mixture 1 except that the mixture contained 0.83 per cent of $l(-)$-histidine. An average of 1.95 γ of $l(-)$-histidine was found per ml. of amino acid test mixture, the percentage of histidine was calculated to be 0.81 per cent, and the histidine recovered was 97.5 per cent. In a series of nineteen analogous experiments the average mean deviation from the mean of the values at the different levels was 3.2 per cent and the recoveries of histidine ranging from 96.1 to 105.6 per cent averaged 100.2 per cent.

ments entirely analogous to those described in earlier papers from this laboratory. These data are given in Tables II to VII.

DISCUSSION

Histidine in Casein

It has been found by assay with *Leuconostoc mesenteroides* P-60 that the present authors' casein contained 2.91 per cent of histidine (uncorrected) and 3.12 per cent of histidine corrected for the 6.21 per cent moisture and

TABLE V

*Results of Assay with Leuconostoc mesenteroides P-60 of Histidine in Silk Fibroin Hydrolysate**

Silk fibroin per tube	Titration volume of 0.046 N NaOH per tube	Histidine found	
		Per tube	Per ml. sample
γ	*ml.*	γ	γ
404	2.04	1.28	1.28
404	2.19	1.45	1.45
808	3.27	2.72	1.36
808	3.29	2.74	1.37
1212	4.18	3.90	1.30
1212	4.30	4.02	1.34
1616	5.10	5.08	1.27
1616	5.11	5.10	1.27
2020	6.18	6.58	1.32
2020	6.09	6.45	1.29

* An average of 1.32 γ of $l(-)$-histidine was found per ml. of silk fibroin hydrolysate. The percentage of histidine in the silk fibroin uncorrected for moisture and ash was calculated to be 0.33 per cent. It was found from twelve comparable assays of the same silk fibroin hydrolysate that the average mean deviation from the mean at the different levels was 2.8 per cent and the percentages of histidine in silk fibroin uncorrected for moisture and ash ranged from 0.31 to 0.35 per cent and averaged 0.32 per cent.

In a series of three experiments the percentages of added histidine recovered from silk fibroin hydrolysates ranged from 101.4 to 106.5 per cent and averaged 103.5 per cent.

0.55 per cent ash reported previously (2). At the present time it seems probable that this value is reasonably accurate (probably within about 5 per cent of the true value) even though it does not agree well with some of the values in the literature.

A summary of nearly all of the values for histidine in casein reported in the literature has been given recently by Vickery and Winternitz (9). It is evident from a consideration of these data that the percentages of histidine obtained by different modifications of the Kossel procedure are from about 15 to 40 per cent lower and that, with one exception, the percentages obtained by colorimetric methods are from about 10 to 30 per cent higher than

TABLE VI

*Results of Assay with Leuconostoc mesenteroides P-60 of Histidine in Amino Acid Test Mixture S**

Amino acid mixture per tube	Titration volume of 0.046 N NaOH per tube	Histidine found	
		Per tube	Per ml. sample
γ	*ml.*	γ	γ
3,126	2.72	1.20	1.20
3,126	2.78	1.26	1.26
6,252	4.60	2.86	1.43
6,252	4.53	2.80	1.40
9,378	6.60	4.40	1.47
9,378	6.48	4.32	1.44
12,504	8.40	5.78	1.44
12,504	8.37	5.76	1.44
15,630	10.00	7.26	1.45
15,630	10.08	7.35	1.47

* The composition of the amino acid test mixture simulating silk fibroin was the same as that given previously (8) except that glutamic acid (7) and lysine (8) were included approximately in the proportions found by the present authors. An average of 1.40 γ of *l*(−)-histidine was found per ml. of amino acid test mixture, the percentage of histidine was calculated to be 0.045 per cent, and the histidine recovered was 100.2 per cent. In a series of seven analogous experiments the average mean deviation from the mean at the different levels was 4.0 per cent and the recoveries of histidine ranging from 95 to 105 per cent averaged 100 per cent.

TABLE VII

*Results of Assay with Leuconostoc mesenteroides P-60 of Histidine Precipitated from Casein Hydrolysate As Its Silver Salt**

Casein hydrolysate aliquot	Histidine			
	Silver salt ppt.	Silver salt filtrate	Total	Hydrolysate
	per cent	*per cent*	*per cent*	*per cent*
A	2.69	0.084	2.77	2.74 (3.00)
A′	2.65	0.080	2.73	
B	2.69	0.085	2.78	2.80 (3.06)
B′	2.67	0.054	2.72	

* Aliquots A and A′ were not subjected to any purification treatment but Aliquots B and B′ were distilled under reduced pressure, decolorized, and filtered. Both aliquots were brought to pH 7.4 for the precipitation of silver histidinate. The pH was determined as accurately as possible with bromothymol blue indicator and buffer solutions checked with the aid of a Beckman pH meter.

Per cent histidine refers to casein uncorrected for ash and moisture. The figures in parentheses refer to per cent histidine in casein calculated to 15.4 per cent nitrogen.

the value reported in this paper. The 2.84 per cent found by Hanke and Koessler (10) in 1920 by colorimetric determination of histidine in the phosphotungstic acid precipitate of the hexone bases was about 8 per cent higher

than the value obtained by Hanke (11) in 1925 by colorimetric determination of histidine in its silver salt precipitate and it is only about 9 per cent lower than that reported in the present experiments.

In 1910 Van Slyke (12) found 3.1 per cent (corrected for solubility of the phosphotungstates) of histidine in casein (containing 15.4 per cent nitrogen) by his nitrogen distribution method and it was stated later (13) that there was no reason why fairly constant results for histidine should not be obtained. Nevertheless, values ranging from 3.4 to 3.9 per cent were reported subsequently by Van Slyke (14) and other investigators (7, 8, 15–17). Van Slyke (14) and Gortner and Sandstrom (18) recovered 90 and 96 per cent of histidine from mixtures containing fourteen and sixteen amino acids, respectively, but no tryptophane, while the latter authors recovered 120 per cent of histidine from a mixture containing tryptophane. It appears, therefore, that the percentage of histidine in casein determined by the nitrogen distribution method may be as much as 20 per cent high. The literature values for histidine in casein, corrected on this basis, would range from 2.7 to 3.1 per cent.

In 1940 Albanese (19) described a procedure in which the basic amino acids were first separated by electrodialysis from other amino acids and humin. Arginine was precipitated as the monoflavianate, excess flavianic acid was removed by electrodialysis, and histidine was precipitated as the mercuric chloride complex. Arginine was calculated from the weight of its monoflavianate, histidine from the nitrogen content of its mercuric chloride complex, and lysine from the nitrogen content of the final filtrate. Tests of the precision and accuracy of this procedure showed that about 99 per cent of arginine and histidine and about 97 per cent of lysine were recovered from a mixture containing eleven amino acids. The percentages in casein (containing 15.4 per cent nitrogen) of histidine (3.1), lysine (8.3), and arginine (3.6) shown in the parentheses were nearly identical with the values for histidine given in this paper and for lysine (4) and arginine (2) reported previously by the present investigators.

A micromethod for the determination of histidine has been described recently by Saidel and Brand (20). Histidine was precipitated from protein hydrolysates with $HgCl_2$, the precipitate was purified and dissolved in HCl, and histidine was determined by colorimetric analysis, with a spectrophotometer, of the product formed by coupling with diazotized sulfanilic acid in the presence of sodium cyanide. Concordant standard values for histidine were obtained with amino acid mixtures simulating the composition of several proteins and, except for β-lactoglobulin, the values found for histidine in proteins were higher than those reported for isolation procedures. Casein was found to contain 2.9 per cent of histidine corrected for moisture and ash.[1]

[1] Personal communication from L. J. Saidel and E. Brand.

The percentage of histidine, 2.53 ± 0.09, in casein (containing 15.75 per cent nitrogen on an ash- and moisture-free basis) was reported recently by Vickery and Winternitz (9) who determined histidine as its bis-3,4-dichlorobenzenesulfonate after precipitating histidine as its silver salt from a hydrochloric acid hydrolysate of the protein. This figure agreed quite well with the values (2.4 to 2.6 per cent) obtained previously by the Kossel and Patten procedure in which histidine was precipitated first as its silver salt and then as its mercuric sulfate complex. It was much higher, however, than the values (1.7 to 1.9 per cent) found by more recent, supposedly improved Kossel procedures. Vickery and Winternitz concluded that "an accuracy of an order of somewhat better than ±3 per cent is to be anticipated with samples of proteins that yield 100 mg. or more of histidine" and that, "On the assumption that the data from the recovery experiments are applicable, the figures are probably within 2 to 3 per cent of the true proportion of histidine yielded by these proteins." It is apparent that all of the experimental manipulations were performed with the utmost care and it is considered that the precision and accuracy of the data reported are higher than any attained previously by the original or any modification of the Kossel method.

The experiments of Vickery and Winternitz can be accepted as evidence that the histidine content of casein is not less than 2.5 per cent. If it were assumed that the true histidine content of casein is 3 per cent, it would follow that the experimental procedure employed by these investigators is subject to some undiscovered error. The only part of the method which was not tested rigorously was the completeness of the precipitation of histidine as its silver salt. It was stated that "reliance is placed . . . upon the complete insolubility of the histidine compound of silver that is formed at the isoelectric point of histidine. No evidence has come to the attention of the writers that suggests that this substance possesses a solubility that is significant in experiments on the scale of these."

It was shown by Vickery and Winternitz that pure histidine was recovered nearly quantitatively from its aqueous solution by direct crystallization of the disulfonate as well as after carrying it through the entire procedure of precipitation as its silver salt and isolation as the disulfonate. Recoveries of comparable accuracy obtained with filtrates from the precipitates of histidine silver in the course of analyses of edestin and casein may not be entirely dependable, however, because of the possibility that appreciable quantities of histidine silver may remain in solution even though all of the added histidine were recovered.

It is of interest in this connection that only from 1.8 to 2.2 per cent of histidine in casein has been found by investigators (21–24) who isolated histidine first as its silver salt and then as its nitranilate. It was reported that 96 per cent of histidine added to a casein hydrolysate was recovered

and that from 90 to 95 per cent of histidine added to the protein prior to hydrolysis was usually recovered.

The following experiment was performed to determine the extent to which histidine is precipitated as its silver salt from a casein hydrolysate under conditions duplicating as exactly as possible those outlined by Vickery and Winternitz. The acid hydrolysate from 25.159 gm. of casein[2] and 300 ml. of concentrated hydrochloric acid was accurately diluted to 500 ml. and silver histidinate was precipitated from four aliquots. The silver in the precipitates and filtrates was removed as silver chloride and the histidine in the original hydrolysate, the filtrates, and the hydrochloric acid solution obtained from the precipitates was determined microbiologically by the method given in this paper. The results of this experiment are given in Table VII.

The average (uncorrected) value, 2.77 per cent, for histidine in the hydrolysate agrees closely with the average value, 2.75 per cent, for total histidine recovered from the four aliquots. It is of interest that this casein preparation calculated to 15.4 per cent nitrogen contained 3.0 per cent histidine, in close agreement with the percentage of histidine found in the casein sample referred to earlier in this paper.

The finding that only about 3 per cent of the histidine in the casein hydrolysate escaped precipitation as the silver salt appears to substantiate the view of Vickery and Winternitz concerning the low solubility of silver histidinate at pH 7.4. On the other hand the 2.92 (corrected) per cent of histidine found by the present authors in the silver histidinate precipitated from a casein hydrolysate (calculated to 15.4 per cent nitrogen) is about 17 per cent higher than the 2.50 per cent which Vickery and Winternitz believe to be correct within ±3 per cent for casein containing 15.75 per cent nitrogen (corrected for ash and moisture).

At the present time it cannot be determined which of these figures for the percentage of histidine in casein is more nearly correct. It has been assumed by some investigators that differences of this magnitude may be accounted for on the hypothesis that casein prepared from different milk samples by different procedures may differ markedly in amino acid composition. While it is true that the non-homogeneity of purified casein has been established by electrophoresis studies, it does not follow, necessarily, that the fractions contain grossly different percentages of any amino acid.

Histidine in Silk Fibroin

The histidine content of silk fibroin was found to be 0.32 per cent, uncorrected, and 0.34 per cent corrected for the 5.68 per cent moisture and

[2] S. M. A. vitamin-free casein containing 8.57 ± 0.03 per cent moisture, 0.93 ± 0.05 per cent ash, and 14.06 (14.07, 13.99, 14.13) per cent nitrogen, uncorrected, and 15.4 per cent nitrogen corrected for moisture and ash.

0.25 per cent ash reported previously (2). Abderhalden (25) found 0.75 per cent and Vickery and Block (26) 0.06 and 0.076 per cent by modified Kossel procedures. It was stated by the latter authors that the figure obtained by Abderhalden probably was high, since it was obtained by calculation from the nitrogen content of the histidine fraction. It was assumed, also, that the figures reported by Vickery and Block were minimal values, since they were obtained by isolating and weighing histidine diflavianate.

SUMMARY

Casein and silk fibroin have been found to contain 3.1 and 0.34 per cent, respectively, of histidine by microbiological assay with *Leuconostoc mesenteroides* P-60. A preliminary study has been made of the precipitation of histidine as its silver salt at pH 7.4 from a casein hydrolysate.

BIBLIOGRAPHY

1. Dunn, M. S., Schott, H. F., Frankl, W., and Rockland, L. B., *J. Biol. Chem.*, **157**, 387 (1945).
2. Dunn, M. S., Camien, M. N., Rockland, L. B., Shankman, S., and Goldberg, S. C., *J. Biol. Chem.*, **155**, 591 (1944).
3. Stokes, J. L., and Gunness, M., *J. Biol. Chem.*, **157**, 651 (1945).
4. Dunn, M. S., Camien, M. N., Shankman, S., Frankl, W., and Rockland, L. B., *J. Biol. Chem.*, **156**, 715 (1944).
5. Dunn, M. S., Shankman, S., Camien, M. N., Frankl, W., and Rockland, L. B., *J. Biol. Chem.*, **156**, 703 (1944).
6. McMahan, J. R., and Snell, E. E., *J. Biol. Chem.*, **152**, 83 (1944).
7. Plimmer, R. H. A., and Lowndes, J., *Biochem. J.*, **31**, 1751 (1937).
8. Jones, D. B., and Gersdorff, C. E. F., *J. Biol. Chem.*, **104**, 99 (1934).
9. Vickery, H. B., and Winternitz, J. K., *J. Biol. Chem.*, **156**, 211 (1944).
10. Hanke, M. T., and Koessler, K. K., *J. Biol. Chem.*, **43**, 527 (1920).
11. Hanke, M. T., *J. Biol. Chem.*, **66**, 489 (1925).
12. Van Slyke, D. D., *Ber. chem. Ges.*, **43**, 3170 (1910).
13. Van Slyke, D. D., *J. Biol. Chem.*, **10**, 15 (1911–12).
14. Van Slyke, D. D., *J. Biol. Chem.*, **16**, 531 (1913–14).
15. Hart, E. B., and Sure, B., *J. Biol. Chem.*, **28**, 241 (1916–17).
16. Crowther, C., and Raistrick, H. O., *Biochem. J.*, **10**, 434 (1916).
17. Dunn, M. S., and Lewis, H. B., *J. Biol. Chem.*, **49**, 327 (1921).
18. Gortner, R. A., and Sandstrom, W. M., *J. Am. Chem. Soc.*, **47**, 1663 (1925).
19. Albanese, A. A., *J. Biol. Chem.*, **134**, 467 (1940).
20. Saidel, L. J., and Brand, E., Abstracts, American Chemical Society, New York, 26B (1944).
21. Block, R. J., *J. Biol. Chem.*, **133**, 67 (1940).
22. Block, R. J., and Bolling, D., The determination of the amino acids, Minneapolis, revised edition, p. iii (1940).
23. Beach, E. F., Bernstein, S. S., Hoffman, O. D., Teague, D. M., and Macy, I. G., *J. Biol. Chem.*, **139**, 57 (1941).
24. Williamson, M. B., *J. Biol. Chem.*, **156**, 47 (1944).
25. Abderhalden, E., *Z. physiol. Chem.*, **120**, 207 (1922).
26. Vickery, H. B., and Block, R. J., *J. Biol. Chem.*, **93**, 105 (1931).

A SIMPLIFIED HYDROGENATION TECHNIQUE FOR THE DETERMINATION OF BLOOD PLASMA TOCOPHEROLS*

By MARY LOUISE QUAIFE AND RAYMOND BIEHLER

(*From the Laboratories of Distillation Products, Inc., Rochester, New York*)

(Received for publication, May 17, 1945)

The chemical method of Quaife and Harris[1] for the estimation of tocopherols in blood plasma employs hydrogenation as a technique for quantitative removal of interfering vitamin A and carotenoids. However, this step is somewhat lengthy for routine analysis and requires equipment not commonly available. Accordingly, we have developed and now use a hydrogenator,[2] to be described in this note, which shortens the time for a single determination of vitamin E in plasma to about 30 minutes. In addition, its applicability to general laboratory hydrogenation on a semimicro scale is suggested and illustrated by results obtained with cinnamic acid.

Fig. 1 shows a drawing of the apparatus. Hydrogen gas from the supply tank is bubbled through the alcohol in Tube A by means of the micro porous disperser tube. The gas, saturated with alcohol vapor, is then bubbled through the sample in Tube B. The exhaust gas passes through a pressure gage and needle valve. By means of the needle valve plus the pressure-regulating valve on the tank, the pressure of gas in the system can be maintained at any desired level.

The revised hydrogenation step is as follows: 10 ml. of the Skellysolve extract of plasma are evaporated to dryness in a 50 ml. conical centrifuge tube and taken up in 10 ml. of ethanol. When the solution is cooled to room temperature, catalyst is added and stirred up thoroughly with a glass rod. The tube is clamped into position and the pressure-regulating valve (two-stage hydrogen reduction valve) on the tank is opened to 15 pounds. The needle valve, which has been closed, is now opened slightly to permit a rapid but smooth flow of gas. This operation is performed quickly so as to maintain a good suspension of catalyst. However, contact between the solution and the rubber joint on the gas delivery tube must be prevented, since ethanol extracts impurities which affect the Emmerie-Engel reagent. After the reduction has proceeded 1 minute, the hydrogen tank valve is closed, atmospheric pressure is restored by gradual opening of the needle

* Communication No. 71 from the Laboratories of Distillation Products, Inc.

[1] Quaife, M. L., and Harris, P. L., *J. Biol. Chem.*, **156**, 499 (1944).

[2] Available through the Vacuum Equipment Division, Distillation Products, Inc., Rochester 13, New York.

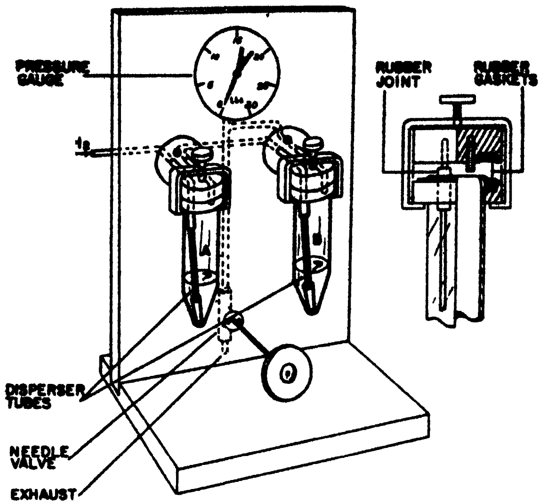


FIG. 1. Semimicro hydrogenation apparatus. See the text for a description of the operation.

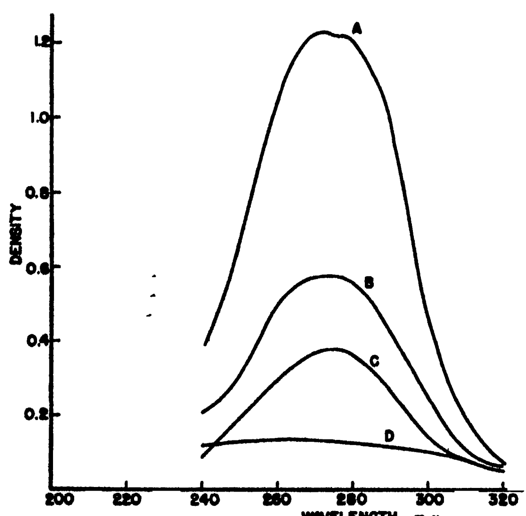


FIG. 2. Effect of pressure on completeness of hydrogenation of cinnamic acid. 1 gm., dissolved in 10 ml. of ethanol, was hydrogenated with 5 per cent Pd-$CaCO_3$ catalyst for 10 minutes at the following pressures: Curve A, control, unhydrogenated; Curve B, 0 pounds; Curve C, 15 pounds; Curve D, 30 pounds. The solutions were diluted to 0.001 per cent for the ultraviolet spectra.

valve, and the tube is removed, corked, and centrifuged. The remainder of the analysis is carried out as described previously.[1]

It was found that the hydrogenated extract tends to be unstable, especially to air, intense light, heat, and continued contact with the activated catalyst. Accordingly, a series of six samples is the suggested number to be hydrogenated successively,[3] followed immediately by centrifugation and completion of the analysis.

Since this new step utilizes one piece of glassware for solvent transfer, hydrogenation, and separation of catalyst, loss of the solution is minimized and it is easy to obtain the 8 ml. aliquot needed for the Emmerie and Engel color reaction.

By the technique outlined above, solutions of crystalline carotene in ethanol were hydrogenated and found to give no reduction of the Emmerie-Engel reagent. The concentration of carotene was greater than would be provided by an original concentration of 1000 γ per 100 ml. of plasma. Further trials showed that saturated solutions of carotene in ethanol are completely decolorized by this procedure.

Solutions of pure α-tocopherol in ethanol, comparable to a plasma level of 1.08 mg. per cent, were hydrogenated similarly and assayed. Average recovery for six samples was 97.8 per cent. Finally, six human plasma samples were analyzed in duplicate by both old and new hydrogenation steps. Good agreement was shown in values for plasma vitamin E obtained by the two techniques. The average difference was ± 1.3 per cent.

Aside from its use in the procedure for determining tocopherol in plasma, the apparatus may be adapted for general laboratory hydrogenations. For example, cinnamic acid was hydrogenated and the progress followed by ultraviolet spectrophotometry.[4] The absorption peak at about 274 mμ disappears with conversion to dihydrocinnamic acid. In one experiment 1 gm. samples of cinnamic acid dissolved in 10 ml. of ethanol were hydrogenated for 10 minutes, according to the technique described, to an extent of about 53 per cent at 0 pounds pressure, 77 per cent at 15 pounds pressure, and 91 per cent at 30 pounds pressure (see Fig. 2). Apparently the degree of hydrogenation is increased as the pressure is increased. Complete reduction can be achieved by using longer hydrogenation times.

SUMMARY

An apparatus suitable for hydrogenation on a semimicro scale is described. By its use the Quaife and Harris procedure[1] for analysis of blood plasma vitamin E is simplified and shortened. Application of the apparatus to general laboratory hydrogenation is suggested and illustrated.

[3] Clean disperser tubes are used for each hydrogenation. They may be reused after an acetone wash, followed by air drying. When the pores of the disperser tubes become clogged on prolonged use, they should be discarded.

[4] An Adam Hilger medium quartz spectrograph with a Spekker photometer was used.

POLAROGRAPHIC STUDIES OF PROTEINS AND THEIR DEGRADATION PRODUCTS

I. THE "PROTEIN INDEX"

By OTTO H. MÜLLER* AND JOHN STAIGE DAVIS, Jr.

(*From the Department of Anatomy, Cornell University Medical College, New York City*)

(Received for publication, May 7, 1945)

In 1933, Brdička (1) discovered that proteins and polypeptides containing cystine or cysteine, when dissolved in a suitably buffered cobalt solution, produce a catalytic reaction during electrolysis at the dropping mercury electrode. This observation became of clinical importance when analyses of blood sera of normal individuals and of persons suffering from cancer revealed significant differences in the height of the polarographic waves resulting from these reactions (2, 3). Like many other tests, the polarographic cancer test unfortunately was not specific; numerous subsequent investigations have demonstrated that serum or plasma proteins in many diseases besides cancer will give abnormal polarographic waves and that occasionally even blood of a person with a malignant disease will fail to be polarographically different from that of a normal individual. Variations and modifications of the original procedure of Brdička so far have failed to improve this condition significantly, but the polarographic protein test has nevertheless proved of value for the study of disease in general.

We were interested in applying this polarographic technique to a study of rheumatoid arthritis and similar diseases and tried out several of the tests proposed by Brdička. Since all of these are empirical, a comparison of the results is impossible unless the experimental conditions are absolutely identical. As a basis of reference, therefore, a "normal" blood must be analyzed simultaneously with each series of unknowns; appreciable variations in the "normals" make this basis rather uncertain. To overcome these difficulties, we developed a new method of expressing the results of such studies, the *protein index*.

The new method is simple and is applicable to routine blood analyses; it is especially designed to avoid the need for rigid temperature control and calibrations of the dropping mercury electrode. One merely determines the ratio of the wave heights obtained in two widely differing empirical tests made on the same blood with the same dropping mercury electrode under identical experimental conditions. After multiplying this ratio by an arbitrary factor, one obtains values which we have called the *protein*

* Present address, Department of Physiology and Pharmacology, University of Nebraska, College of Medicine, Omaha 5, Nebraska.

index. Since the two tests chosen for this analysis show deviations for abnormal blood which are usually in opposite directions, the resulting index magnifies these deviations from the normal and thus improves the sensitivity of the analysis. It will be shown in this paper that, within the range observed with normal and abnormal bloods, the index values are but little influenced by the usual variations in "room" temperature and by considerable changes in the drop size and drop time of the dropping mercury electrode. It is therefore possible to characterize a given blood by its protein index and thus give a quantitative expression of its deviation from a standard. Variations in the protein indexes of the blood of normal individuals and patients and of some animals will be discussed in subsequent papers in which evidence will also be presented that the protein index of any given "normal" individual is remarkably constant over long periods of time. This new method, therefore, eliminates the necessity of simultaneous analyses of a "normal" with an unknown blood.

Besides illustrating the limits of applicability of the protein index, the studies on the effect of drop time and drop size on the catalytic protein waves brought out a relationship which had not been recognized before. As will be shown below, the height of these waves is practically independent of changes in the drop time of a given electrode but seems to be proportional to the surface area of the drops or to the two-thirds power of the radius of the capillary orifice.

Determination of Protein Index

The two tests we used in determining the protein index are modifications of some of Brdička's procedures (2–4). To distinguish them readily, we have called them *digest* and *filtrate* tests respectively. The possibility of further refinement in these tests is anticipated, but should not alter the value of the principle of measurement which is proposed in this and subsequent papers. We rigidly adhered to the following procedures.

Digest Test—To 0.50 cc. of oxalated plasma are added 0.50 cc. of water and 0.25 cc. of 1.0 N potassium hydroxide. This solution is thoroughly mixed and left standing at room temperature for 30 minutes. Then 0.05 cc. of the alkaline digest is added to 10.0 cc. of a buffered cobalt solution and polarographed immediately in an open beaker, starting at −0.8 volt *versus* the saturated calomel electrode. The buffered cobalt solution must be freshly prepared and consist of 1.6×10^{-3} M cobaltous chloride, 0.1 N ammonium chloride, and 0.1 N ammonium hydroxide (care must be taken to add the ammonium hydroxide last, to prevent precipitation of some of the cobalt).

Filtrate Test—To 0.50 cc. of oxalated plasma are added 1.00 cc. of water and 0.10 cc. of 1.0 N potassium hydroxide. After this has stood for 30

minutes at room temperature, 1.00 cc. of 20 per cent sulfosalicylic acid is added and the mixture is vigorously shaken. Exactly 10 minutes after the addition of the protein precipitant, the suspension is poured through a Whatman No. 5 filter paper (5.5 cm. in diameter). The resulting clear filtrate is fairly stable and can be analyzed when convenient. 0.50 cc. of this filtrate is added to 5.0 cc. of a solution consisting of 0.001 M hexammonium cobaltic chloride, 0.1 N ammonium chloride, and 0.8 N ammonium hydroxide. (Because of the volatility of the ammonia, the latter solution must be kept in a well stoppered bottle and should be pipetted but a few minutes before the filtrate is added.) The mixture is polarographed immediately, in an open beaker, starting at −0.8 volt (*versus* the saturated calomel electrode).

In the digest test, as a rule, the height of the catalytic wave becomes smaller the greater the deviation of the blood from "normal," while the opposite is true for the filtrate test. In our new method, the wave height obtained in the filtrate test is divided by that found in the digest test for the same blood sample under identical experimental conditions. This ratio, multiplied by a suitable factor, gives us the *protein index* which is used to compare different blood samples; it becomes larger the greater the deviation of the blood from normal.

The purpose of multiplying the ratio by a factor is to get whole numbers for the protein index, starting with unity for the lowest "normal" value. This factor is 15 if the wave heights are expressed in microamperes or, as read directly off the polarogram, if they are expressed in mm. and the galvanometer sensitivities used in the two tests are the same. However, if the filtrate wave is recorded at a higher galvanometer sensitivity than the digest wave, as is more often the case, the factor will include a correction for this difference in sensitivity and be smaller (*e.g.*, if the digest wave is taken at 1/150 and the filtrate wave at 1/100 of maximum galvanometer sensitivity, the factor is 10).

To illustrate this method, a polarogram showing both digest and filtrate tests of three sufficiently different plasmas is reproduced in Fig. 1. This and all other polarograms were recorded with a Heyrovský-Shikata polarograph, Nejedlý's model VIII. The galvanometer used had a maximum sensitivity of 2.1×10^{-9} amperes per mm. at 1 meter. A saturated calomel half-cell served as a non-polarizable electrode; it was connected to the test solutions by means of potassium chloride and potassium nitrate agar bridges.[1]

The first wave in each of the curves of Fig. 1 is due to the reduction of cobalt, which results in a steep maximum in the absence of a maximum sup-

[1] For a discussion of the principles of polarography and of the terms used see the monographs of Heyrovský (5), Kolthoff and Lingane (6), and Müller (7).

pressor (1, 5–7). The presence of protein in the digests fully suppresses this maximum and leads to a short horizontal portion in the curve which can be used as a convenient base-line for measuring the height of the protein *double wave* which follows (see Curves A, B, C, Fig. 1). The protein degradation products left in the filtrate are less effective in suppressing maxima; only when they are present in relatively high concentrations do they suppress the cobalt maximum entirely (compare Curve *a*, Fig. 1, with Curves *b* and *c*). Since this situation is rare and no other satisfactory suppressor for this maximum is known,[2] we have used as a base-line the height of the cobalt wave found with the test solution in the absence of filtrate (not shown in Fig. 1). The wave heights of the *double wave* are then measured always to the second peak of this wave.

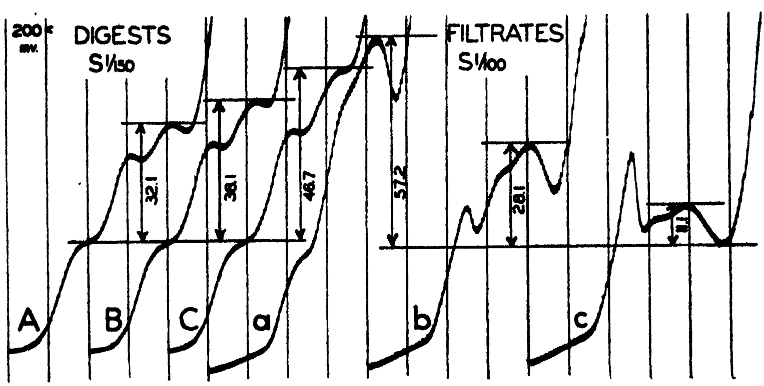


FIG. 1. Polarogram showing digest curves (capital letters) and filtrate curves (lower case letters) of plasmas from (*a*) a dog with severe infection, (*b*) a dog with mild infection, and (*c*) a normal human being.

It may be seen from Fig. 1 that the smallest digest wave (Curve A) is obtained with the plasma which gives the largest filtrate wave (Curve *a*), while the largest digest wave (Curve C) corresponds to the smallest filtrate wave (Curve *c*). Since the galvanometer sensitivities used in this case were 1/150 and 1/100, the ratio of filtrate to digest wave must be multiplied by 10, to give the following protein indexes: (*a*) = 17.8, (*b*) = 7.4, (*c*) = 2.4. These results are significant to the first decimal when the experi-

[2] Winzler, Burk, and Hesselbach (8) used caffeine to "suppress oxygen and cobalt maxima" in a solution very similar to the filtrate test solution. We cannot recommend this procedure because (*a*) there is no need to suppress the oxygen maximum, (*b*) in the concentrations used, caffeine not only lacks any effect on the cobalt maximum but may actually suppress the double wave, and (*c*) at higher concentrations, the caffeine definitely suppresses the double wave, while the cobalt maximum is but slightly affected.

mental conditions are sufficiently constant. It will now be shown that such values are still good to the nearest whole number during small fluctuations of the room temperature and when obtained with capillaries of differing characteristics. Classification of different bloods by whole numbers is quite adequate at present, since in our experience the protein indexes may range from 1 to 4 for "normal" and up to 20 for abnormal human plasmas.

Effect of Temperature

Because a catalytic reaction is involved, the effect of temperature on the *double wave* is unusual, and its careful study may contribute significantly to an understanding of this reaction (9). However, the instability, especially at elevated temperatures, of both the proteins and the test solutions in which they are examined renders such an investigation extremely difficult. For purposes of orientation, we have made some crude experiments which seem adequate for indicating the magnitude of the errors involved in insufficient temperature control.

In Figs. 2 and 3 are reproduced typical polarograms of the digest and filtrate tests, respectively. Each solution was kept in a small open beaker, partially immersed in water contained in a larger vessel. The temperature of this water bath could be changed quickly and the curves were recorded as soon as the temperature of the test solution was within 1° of that of the bath. In spite of all haste, about an hour elapsed between the recording of the first (at the lowest temperature) and the last curve, during which time the solution had altered considerably. To get a notion of the magnitude of this change, each solution had been analyzed immediately before this experiment at room temperature (25°).

As may be seen from Figs. 2 and 3, the most pronounced effect of temperature is on the first component of the double wave. At lower temperatures, this wave decreases out of proportion to the rest of the waves and seems to disappear entirely in the filtrate test at 1°. On the other hand, we have found that at temperatures above 30° the first portion of the double wave in the filtrate test becomes even larger than the second portion. Since the first catalytic wave can be made to overlap with the second in the digest test by a 10-fold increase in the ammonium hydroxide concentration of the test solution, we feel that this first catalytic wave is largely controlled by the activity of free ammonia in the solution which is increased at higher temperatures.

Fortunately temperature has a much smaller effect on the second portion of the double wave which we measure to get the protein index. Inspection of Figs. 2 and 3 reveals that the height of this wave shows a consistent increase with temperature in the digest test, while in the filtrate test a definite increase with temperature below 16° is followed by a decrease in the wave

height above this temperature. Unquestionably most of this decrease is caused by decomposition of the solution; the fact that the wave cannot be further increased by a rise in temperature suggests that the final rate-limiting reaction must be independent of temperature. By comparing appropriate curves of these polarograms with those obtained in the pre-

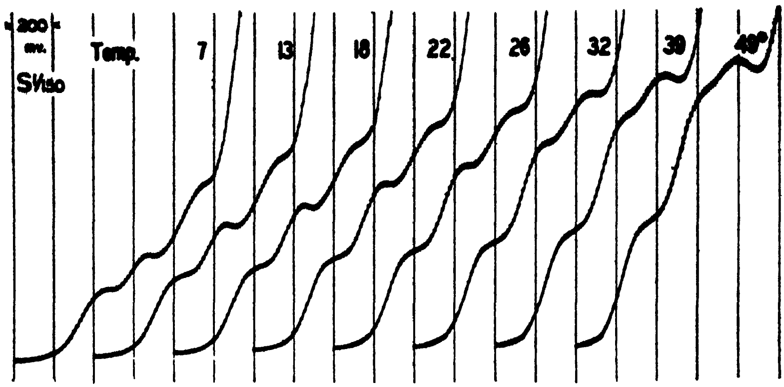


FIG. 2. Polarogram showing the effect of temperature on digest curves of normal human plasma.

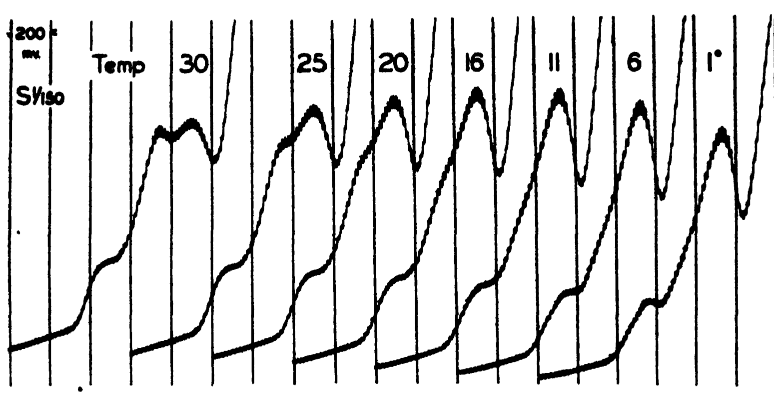


FIG. 3. Polarogram showing the effect of temperature on filtrate curves of plasma from a patient with carcinoma.

liminary runs carried out at 25°, we were able to ascertain that the digest wave had decreased 5.3 per cent and the filtrate wave 14.3 per cent up to the time that these curves were recorded. The greater decrease in the filtrate wave is caused by the more rapid loss of ammonia from this more concentrated solution at higher temperatures.

In order to get an idea about the magnitude of the effect of variations in

room temperature on the protein index we had to make a number of crude approximations. First, we corrected the wave heights observed between 16–32° to the most reasonable figures based on the measured decomposition at 25°. Then we calculated the protein index, multiplying the ratio of filtrate to digest by 15. Although the plasmas and the dropping mercury electrodes used in the two tests were not the same, this procedure is justifiable because the values thus obtained approximate the highest ever observed by us in actual analyses of human plasmas, and thus permit us to estimate the temperature effects when they are largest. The data involved in these calculations are shown in Table I. From these it can be seen that higher indexes may be expected at lower temperatures, and that a range of room temperatures from 21–26° could cause deviations as high as two units

TABLE I

Effect of Temperature on the Protein Index

Data from Figs. 2 and 3.

Temperature	Wave height				Ratio, $\frac{\text{filtrate}}{\text{digest}}$	Protein index
	Observed		Corrected			
	Digest	Filtrate	Digest	Filtrate		
°C.	mm.	mm.	mm.	mm.		
16		50.8	34.5	52.7	15	23
18	33.0					
20		47.5				
21			36.0	51.6	14	21
22	35.5					
25		43.2				
26	37.4		39.5	50.0	13	19
30		38.2				
31			42.7	48.5	11	17
32	40.0					

in the protein index if the latter has a value of about 20. This deviation obviously is less when the protein index is smaller and it is furthermore counteracted by an opposite effect of temperature on the speed of denaturation of the proteins in an alkaline solution. For instance, identical solutions of plasma plus potassium hydroxide, when left standing for 30 minutes each at 5°, 14°, 26°, and 37.5°, and then added to a cobalt buffer at 26° and polarographed, gave protein double waves which were 54.7, 54.4, 46.6, and 39.5 mm., respectively, in height. This balancing of temperature effects could be further demonstrated on a human plasma with a high protein index, which was analyzed on two different days at room temperatures of 23° and 26°. The wave heights obtained in the filtrate and digest tests were 75.6 and 68.2 mm. at 23° and 75.5 and 67.8 mm. at 26°. Multiplying

the filtrate to digest ratios by 10, one gets index values of 11.10 and 11.12 respectively. We may conclude, therefore, that *small fluctuations in room temperature do not affect the values of the protein index significantly.*

Effect of Drop Time and Drop Size of the Dropping Mercury Electrode

The effect of concentration of the reacting material and of the drop time and drop size of the electrode on the *diffusion current,* obtained during the direct reduction of electroactive material, can be predicted from the Ilkovič equation (10). Unfortunately, this equation is not applicable to *catalytic currents* of the type observed in these protein reactions. Brdička (11) was able to derive an equation, based on Langmuir's adsorption isotherm, which expressed fairly well the relationship between catalytic current and concentration. However, the determination of the three constants contained in this equation is so complicated and time-consuming that it is out of the question for routine work. Experiments carried out in this laboratory have shown furthermore that these constants vary with different plasmas and proteins and with different experimental conditions (see also Brdička (11) and Heyrovský (5)).

There are no quantitative data in the literature concerning the effect of drop time and drop size on the height of the catalytic protein wave. This problem was consequently studied in some detail. To illustrate the results by a typical example, two polarograms have been reproduced in Fig. 4. They show a digest and a filtrate test of the same human plasma, obtained with two different dropping mercury electrodes under varying pressures. The evaluation of these curves and the calculations based thereon are given in Table II. These data are uncorrected for the inevitable deterioration of the solutions with time (it took 30 and 40 minutes to prepare the two polarograms), the magnitude of which again was ascertained by a suitable preliminary experiment. The filtrate curves were recorded, starting with an applied voltage of zero (*versus* the saturated calomel electrode) instead of −0.8 volt, so that the second step in the reduction of the trivalent cobalt (at −1.0 volt) could be measured with greater certainty; the first step in this reduction is masked by the oxygen maximum (at −0.1 volt) with which it overlaps.

All currents were measured in two different ways indicated on some of the curves of Fig. 4 (to preserve the clarity of the polarograms, the wave heights are not drawn in for all of the curves). The results are given in Table II. We first followed the customary procedure of evaluating the protein waves; namely, measuring the *maximum* of the galvanometer excursions as in Fig. 4, Curve *a*. Since measurement of the *average* of the galvanometer oscillations has yielded the theoretically most satisfactory values when regular diffusion currents are involved (12), the currents were

also measured in this fashion (see Fig. 4, Curve *e*). To distinguish the data, values obtained by the latter method are given in bold-faced type in Table II. The accuracy of these measurements is somewhat less than that customary in determining diffusion currents, mainly because of uncertainties about the complete suppression of the cobalt wave in the filtrate test. However, the results are sufficiently consistent in themselves and

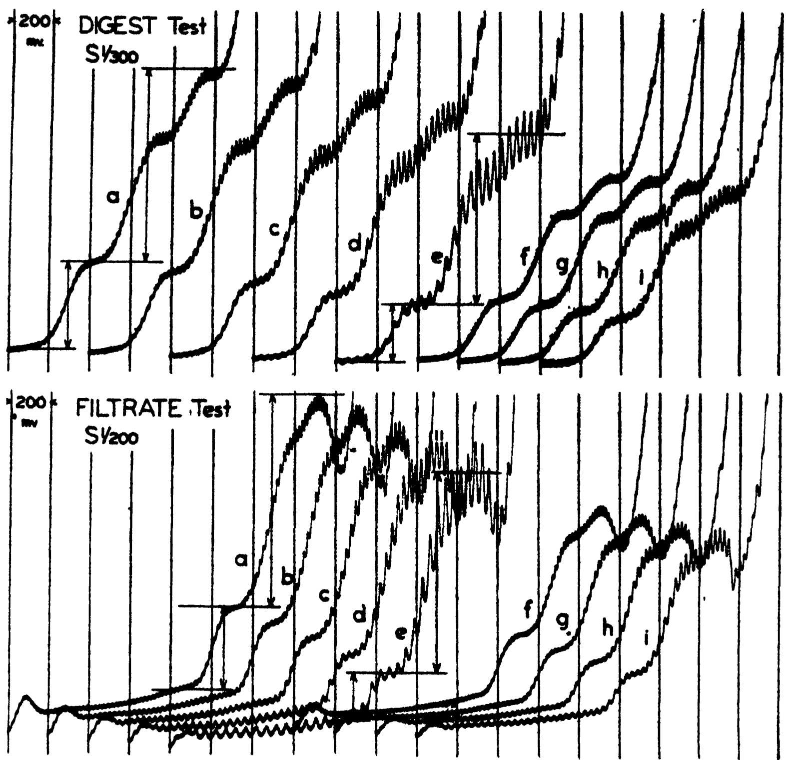


FIG. 4. Polarograms showing the effect of drop time and drop size on digest and filtrate curves of plasma from a patient with carcinoma. (The experimental data are given in Table II.)

agree with other similar determinations well enough to warrant a careful scrutiny.

As has been shown by Müller (13), the following two constants fully characterize a given capillary: ρ, the radius of the capillary orifice and κ, the capillary constant (the pressure of mercury, in cm., necessary to force 1 mg. of mercury through the capillary per second). These constants were determined for the two capillaries used in the preparation of Fig. 4, and are given in Table II. Knowing ρ, we were able to calculate the *back pressure*

(13) and thus correct the *apparent* pressures listed in Table II. The corrected pressures, divided by κ, then give us the values for m, the flow of mercury in mg. per second (13).

TABLE II

Effect of Drop Time and Drop Size on Digest and Filtrate Waves and on Protein Digest

	Curve (Fig. 4)	*a*	*b*	*c*	*d*	*e*	*f*	*g*	*h*	*i*
	Capillary No.	1 (κ = 26.6, ρ = 27.2 μ)					2 (κ = 46.7, ρ = 14.9 μ)			
	Apparent pressure, *cm.*	70.0	60.0	50.0	40.0	30 0	74.0	63.0	53.5	43.5
	m, *mg. per sec.*	2.57	2.19	1.82	1.44	1.06	1.54	1.31	1.10	0.89
Digest test	t, *sec.*	2.7	3.2	3.8	4.8	6.6	2.5	2.9	3.4	4.3
	Cobalt wave, *mm.*	23.6	21.8	20.2	18.1	16.2	15.9	14.8	13.5	12.5
	" " $/m^{2/3}t^{1/6}$	10.7	10.7	10.9	11.0	11.4	10.3	10.3	10.4	10.8
	Double " *mm.*	51.7	51.7	51.0	50.6	50.4	33.5	34.4	34.7	34.8
	" " $/m^{2/3}t^{2/3}$	14.2	14.1	14.2	13.9	13.8	13.6	14.1	14.4	14.3
	" " $/\rho^{2/3}$	5.7	5.7	5.6	5.6	5.6	5.5	5.7	5.7	5.7
	Cobalt " *mm.*	**23.3**	**21.5**	**19.6**	**17.5**	**15.3**	**15.9**	**14.8**	**13.4**	**12.4**
	" " $/m^{2/3}t^{1/6}$	**10.5**	**10.5**	**10.5**	**10.6**	**10.8**	**10.3**	**10.4**	**10.3**	**10.7**
	Double " *mm.*	**50.8**	**50.4**	**49.4**	**47.8**	**45.9**	**32.7**	**33.9**	**33.7**	**33.6**
	" " $/m^{2/3}t^{2/3}$	**14.0**	**13.7**	**13.6**	**13.1**	**12.6**	**13.3**	**13.9**	**14.0**	**13.8**
	" " $/\rho^{2/3}$	**5.6**	**5.6**	**5.5**	**5.3**	**5.1**	**5.4**	**5.6**	**5.5**	**5.5**
Filtrate test	t, *sec.*	2.8	3.2	3.9	4.9	6.7	2.5	2.9	3.5	4.3
	Cobalt wave, *mm.*	22.4	20.2	17.8	15.2	13.0	16.8	14.7	12.6	10.9
	" " $/m^{2/3}t^{1/6}$	10.0	9.9	9.6	9.2	9.1	10.8	10.3	9.7	9.4
	Double " *mm.*	57.4	58.1	57.7	59.6	60.7	35.2	36.1	37.1	38.5
	" " $/m^{2/3}t^{2/3}$	15.4	15.8	15.5	16.1	16.5	14.3	14.8	15.5	15.9
	" " $/\rho^{2/3}$	6.3	6.4	6.4	6.5	6.7	5.8	5.9	6.1	6.3
	Cobalt " *mm.*	**22.6**	**20.3**	**18.1**	**15.6**	**13.0**	**17.2**	**15.0**	**12.9**	**11.2**
	" " $/m^{2/3}t^{1/6}$	**10.0**	**10.0**	**9.7**	**9.4**	**9.1**	**11.1**	**10.5**	**9.9**	**9.7**
	Double " *mm.*	**55.4**	**55.9**	**54.6**	**55.0**	**53.9**	**34.0**	**34.8**	**35.4**	**36.0**
	" " $/m^{2/3}t^{2/3}$	**14.9**	**15.2**	**14.7**	**14.9**	**14.6**	**13.8**	**14.2**	**14.7**	**14.8**
	" " $/\rho^{2/3}$	**6.1**	**6.2**	**6.0**	**6.1**	**6.0**	**5.6**	**5.7**	**5.8**	**5.9**
Protein index	Maximum galvanometer excursions	11.1	11.2	11.3	11.7	12.0	10.5	10.5	10.7	11.1
	Average galvanometer excursions	**10.9**	**11.1**	**11.1**	**11.5**	**11.7**	**10.4**	**10.3**	**10.5**	**10.7**

κ = capillary constant; ρ = radius of the capillary orifice; m = flow of mercury; t = drop time.

The bold-faced figures represent the values obtained by measurement of the average galvanometer excursions.

The drop time, t, was determined while a constant voltage of −0.8 (*versus* saturated calomel electrode) was applied to the electrode. These measurements were made after the polarograms were recorded to prevent

delay and excess deterioration of the solutions during the recording. The values thus obtained were almost identical for different digests and filtrates, but were significantly smaller than in the pure cobalt buffers, thus illustrating the presence of surface-active material (5–7). Hence, it is not strictly correct to use the cobalt wave height in the pure solution as a base-line for the double wave, as we have done; yet it is the best method available because the error produced thereby is constant and does not affect our results.

In looking over the polarograms of Fig. 4 and the data of Table II, one can see clearly that lowering the pressure on a given electrode produces a definite decrease in the cobalt wave, while it has practically no effect on the height of the double wave. On the other hand, a change from the faster to the slower electrode may cause very little alteration of the cobalt wave height but may reduce the height of the catalytic wave by almost one-half.

In spite of these marked differences in the catalytic currents observed with the two electrodes and in spite of the wide range of drop times employed, we find that the protein index (obtained by multiplying the filtrate to digest ratio by 10) is altered very little. There seems to be no advantage in using average rather than maximum galvanometer deflections, and we recommend continuation of the latter, simpler method of measuring catalytic currents.

The tendency towards larger index values with an increased drop time is real, since it becomes more pronounced if we correct for the deterioration of the solution. The preliminary analysis made with Capillary 2 at an apparent pressure of 74 cm. gave filtrate and digest waves 37.8 and 33.9 mm., respectively, in height, yielding a value of 11.1 for the protein index. This agrees very well with that found in the first analysis made with Capillary 1 (Curve *a*), but it is 0.6 unit higher than the corresponding wave made about 20 minutes later with Capillary 2 (Curve *f*). Hence, it is probable that the protein index of Curve *e* would be about 12.5 if the solution had not deteriorated. This shows that we may expect errors in a protein index of this magnitude amounting to a whole unit when very slow capillaries are used. Similar experiments have proved that this error is smaller when the protein index has a lower value, and that it is negligible at drop times of 1.5 seconds. Thus we are led to conclude that *the protein index is practically independent of the capillary characteristics as long as the drop time remains between 1.5 and 3.5 seconds.*

Table II further illustrates that the changes in the height of the cobalt wave are typical of a regular diffusion current; the ratio between the wave height and the quantity $m^{2/3}t^{1/6}$ is fairly constant, as required by the Ilkovič equation (10). It may be further noted that this agreement with the

Ilkovič equation is better when the *average* galvanometer deflections are measured (12).

The fact that changes in drop time of a given electrode have such little effect on the double wave suggested that the surface area of the mercury drop is the determining factor, since this remains practically uninfluenced by pressure changes on the capillary (5–7). This surface area, A, may be related to W, the weight of the drop, or to the product of m, the rate of flow of mercury, and t, the drop time, or to ρ, the radius of the capillary orifice (13), by the following equations,

$$A = k \cdot W^{2/3} = k \cdot m^{2/3} t^{2/3} = k \cdot \left(\frac{2\pi\sigma}{g}\right)^{2/3} \cdot \rho^{2/3}$$

where k is a proportionality constant, σ is the surface tension of mercury in dynes per cm. and g is the acceleration of gravity. As may be seen from Table II, the double wave to $m^{2/3}t^{2/3}$ ratio is approximately constant, thus providing the means for a comparison of the catalytic protein waves when obtained with different electrodes.

If we neglect small changes in surface tension, we can also use the ratio of the double wave to $\rho^{2/3}$ for this purpose (see Table II). As has been pointed out earlier in this paper, the drop time varied but little in a number of different filtrates or digests; consequently, surface tension of the mercury, which is a factor determining this drop time (13), must also remain practically constant. Thus a knowledge of ρ is sufficient to make possible a fair comparison of double waves obtained with electrodes of different characteristics.

SUMMARY

The protein index is proposed as a convenient method of characterizing and comparing polarographic results obtained with blood proteins and their degradation products during reduction in a buffered cobalt solution. It represents the ratio of the two wave heights obtained in the filtrate and the digest tests, multiplied by a suitable factor. The protein index is practically independent of small variations in room temperature and of considerable changes in the drop time and drop size of the dropping mercury electrode, and thus is especially well suited for routine analysis of blood proteins.

The height of the protein double wave is little influenced by alteration in the drop time of a given electrode, and it is approximately proportional to the surface area of the mercury drops or to the two-thirds power of the radius of the capillary orifice.

The authors gratefully acknowledge their indebtedness to Mr. Howard Phipps, Mrs. Joan W. Payson, and Mr. Marshall Alworth for financial support of this investigation.

BIBLIOGRAPHY

1. Brdička, R., *Collect. Czechoslov. Chem. Communicat.*, **5**, 112 (1933).
2. Brdička, R., *Nature*, **139**, 330 (1937).
3. Brdička, R., *Acta Union internat. cancer*, **3**, 13 (1938).
4. Brdička, R., Novák, F. V., and Klumpar, J., *Acta radiol. et cancerol. Bohem. et Morav.*, **2**, 27 (1939).
5. Heyrovský, J., Polarographie, Vienna (1941).
6. Kolthoff, I. M., and Lingane, J. J., Polarography, New York (1941).
7. Müller, O. H., The polarographic method of analysis, Easton (1941).
8. Winzler, R. J., Burk, D., and Hesselbach, M., *J. Nat. Cancer Inst.*, **4**, 417 (1944).
9. Müller, O. H., *Am. J. Physiol.*, **133**, 393 (1941).
10. Ilkovič, D., *Collect. Czechoslov. Chem. Communicat.*, **6**, 498 (1934).
11. Brdička, R., *Collect. Czechoslov. Chem. Communicat.*, **11**, 614 (1939).
12. Müller, O. H., *J. Biol. Chem.*, **145**, 425 (1942).
13. Müller, O. H., *J. Am. Chem. Soc.*, **66**, 1019 (1944).

SPECTROPHOTOMETRIC STUDY OF A NEW COLORIMETRIC REACTION OF VITAMIN A*

By ALBERT E. SOBEL and HAROLD WERBIN

(*From the Pediatric Research Laboratory, The Jewish Hospital of Brooklyn, New York*)

(Received for publication, February 2, 1945)

This paper deals with a new colorimetric reaction of vitamin A with glycerol 1,3-dichlorohydrin (which we henceforth will refer to as GDH) that appears to be suitable for the quantitative estimation of this vitamin.

The estimation of vitamin A *in vitro* has been the subject of many reviews in recent years (1–6). The two widely used methods at present are the ultraviolet absorption at 325 to 328 mμ, and the determination of the maximum absorption at 615 to 620 mμ of the blue color formed on the addition of a solution of antimony trichloride in chloroform to the vitamin in the same solvent (Carr-Price reaction).

One disadvantage of the ultraviolet method is that there are substances in natural products other than vitamin A which absorb at around 328 mμ (7). Another drawback of the method is the need for expensive equipment.

The Carr-Price reaction does not require expensive equipment and is more specific for vitamin A (8). Its disadvantages are the rapidity with which the maximum absorption must be read, owing to the fading of the color, and the instability of the reagent.

The new colorimetric reaction reported herein possesses the same advantages that the Carr-Price reaction has over the ultraviolet absorption method, and in addition possesses the following advantages over the Carr-Price reaction. (1) The stability of the color produced permits its measurement with ease any time from 2 to 10 minutes after the reagents are mixed. (We have even been able to measure the color in a visual colorimeter.) (2) The reagent employed is stable. It is not affected by traces of moisture and leaves no film which might interfere in the absorption of the color solution. The disadvantage of the new reaction is that the extinction coefficient[1] ($L^{1\%}_{1\,\text{cm.}}$, λ 550 mμ) of the color produced is about one-fourth that of the antimony trichloride blue color ($L^{1\%}_{1\,\text{cm.}}$, λ 615 mμ).

* This study was aided by a grant from the United Hospital Fund of the city of Greater New York. Presented before the American Society of Biological Chemists (*Federation Proc.*, **4**, 104 (1945)).

[1] The term $L^{1\%}_{1\,\text{cm.}}$, introduced by Dann and Evelyn (9) for use in photoelectric colorimeters which employ a band of light of about 30 to 40 mμ wide, has been used by many authors (10–12). The term is analogous to $E^{1\%}_{1\,\text{cm.}}$, the latter being used with those instruments which employ monochromatic light. In this paper, to the

The reagent used in the authors' reaction is the practical grade of GDH obtained from the Eastman Kodak Company. The keeping of this reagent requires no special precautions. One simply uses it as it comes from the bottle.

Upon the addition of this reagent to a solution of vitamin A in chloroform, an immediate blue color appears which rapidly changes into a more stable color resembling a dilute solution of potassium permanganate.

Fig. 1 shows the absorption curve of the immediate blue color. The absorption maximum is at 625 mμ. The shape of the curve is similar to the

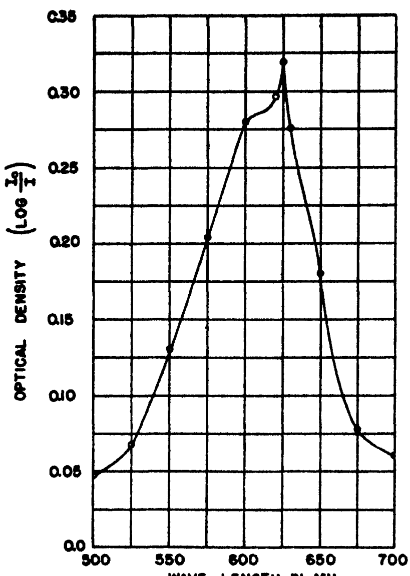


FIG. 1. Absorption curve of the immediate blue color produced by vitamin A and glycerol 1,3-dichlorohydrin. The solution contained 33 84 I.U. of vitamin in 5.0 ml. of solution.

one obtained with antimony trichloride and vitamin A, but is not identical to it. The blue color formed with GDH appears to obey Beer's law up to 33 I.U. of vitamin A in 5.0 ml. of solution, but a complete study was not made of this point in view of the stability of the second color formed.

Fig. 2 shows the absorption spectrum of the violet color developed 2 minutes after the reagents were mixed. The maximum absorption occurs

authors' knowledge, $L^{1\%}_{1\,\text{cm}}$, has been used for the first time with data obtained on a Coleman model 11 spectrophotometer because, although it is constructed with a diffraction grating rather than a light filter, it has a wave band of light 35 mμ wide.

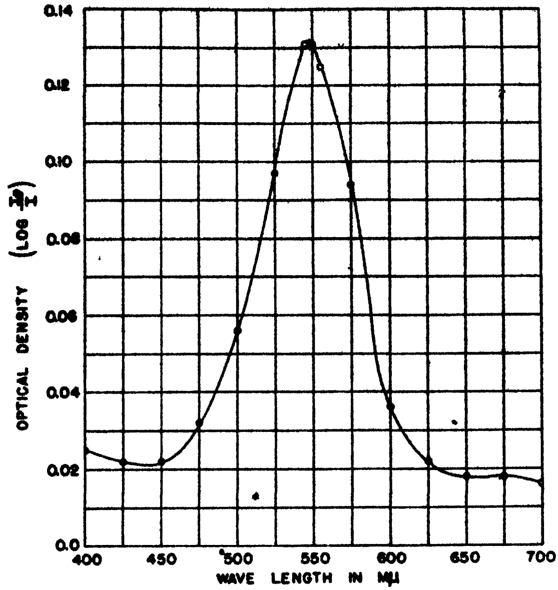


FIG. 2. Absorption curve of the violet color produced by vitamin A and glycerol 1,3-dichlorohydrin at the end of 2 minutes. The solution contained 16.92 I.U. of vitamin in 5.0 ml. of solution.

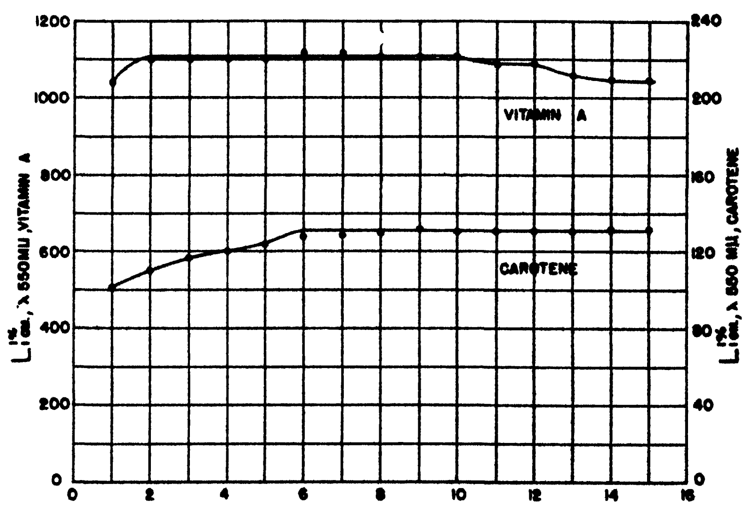


FIG. 3. Time curve of the changes in $L^{1\%}_{1\ \mathrm{cm.}}$ at 550 mμ of the colors produced by 6.76 γ of vitamin A and 60.08 γ of carotene (90 per cent of β- and 10 per cent of α-) with glycerol 1,3-dichlorohydrin at 25°. The total volume of reaction mixture in each case was 5.0 ml.

at 550 mμ, and remains constant at 25° for from 2 to 10 minutes. After that time a slow deterioration of the color takes place, as can be seen in Fig. 3.

In Fig. 4 is presented the relationship between light absorption and concentration at 550 mμ. Beer's law is obeyed up to a concentration of 25 I.U. of vitamin A in 5.0 ml. of solution. Above this value the absorption is slightly less than that to be expected from Beer's law. Under the experimental conditions we employed, the blue color of antimony trichloride obeyed Beer's law up to a concentration of 23 I.U. in 4.0 ml. of solution.

The $L^{1\%}_{1\,cm.}$ at 550 mμ for vitamin A in the concentrate employed was 1010. (In this calculation 1.00 I.U. equals 0.292 γ of vitamin A. The derivation of this factor is explained in the experimental portion of this

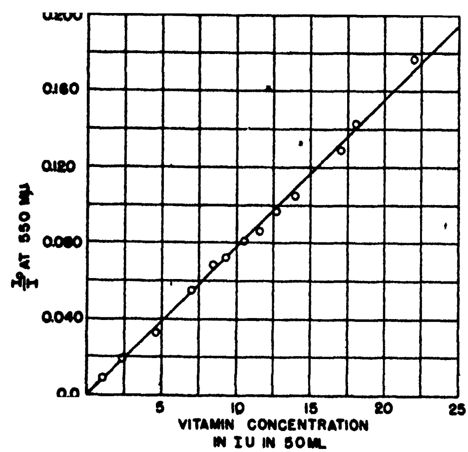


FIG. 4. Relationship between absorption at 550 mμ and the vitamin concentration.

paper.) The $L^{1\%}_{1\,cm.}$ of the immediate blue color at 625 mμ with GDH was 1385. The $L^{1\%}_{1\,cm.}$ obtained at 615 mμ with antimony trichloride was 3900. This compares with the $L^{1\%}_{1\,cm.}$ at 622 mμ of 3880 for crystalline vitamin A (12).

To test the interference of substances found in fish liver oils upon the new colorimetric reaction, the vitamin A content of several fish liver oils was determined with GDH and antimony trichloride. The results are presented in Table I. The antimony trichloride method is one of the accepted procedures for the estimation of vitamin A in fish liver oils, and according to Oser *et al.* (8) the results are in closer agreement with those by the multiple level bioassay than with those obtained by the direct spectrophotometric method in the ultraviolet.

The agreement between the GDH and antimony trichloride methods is close. That the values obtained do not coincide may be ascribed to the differences in the interference upon the colors produced by the two reagents with vitamin A caused by the other components of the oil. A more exhaustive study will be undertaken in order to determine which method is more specific for vitamin A.

The $L^{1\%}_{1\,cm.}$ values, λ 550 mμ, for vitamins D_2 and D_3, ergosterol, 7-dehydrocholesterol, and cholesterol, after 15 minutes, otherwise the conditions being the same as in the estimation of vitamin A, were 11.28, 5.96, 1.13, 0.08, and 0.00 respectively. These values (obtained from Sobel, Mayer, and Kramer (15)) at 2 minutes (the time recommended for the development

TABLE I

Comparison of Vitamin A Values Obtained on Fish Liver Oils by Glycerol 1,3-Dichlorohydrin and $SbCl_3$ Methods

The values are expressed as international units of vitamin A per gm. of oil.

Type of oil	Glycerol 1,3-dichlorohydrin	$SbCl_3$	$[E^{1\%}_{1\,cm.}] \times [2000]$ at 328 mμ*
Oil 1, whole	24,200	27,000	28,200
" 1, unsaponifiable fraction†	24,000	25,000	
" 2, whole	8,070	8,240	10,200
" 2, unsaponifiable fraction†	8,040	7,370	
" 3, whole	36,500	37,100	44,000
" 5, "	73,300	74,900	85,000
Concentrated Oil G‡	1,285,000	1,204,000	1,240,000
" " I‡	1,013,000	973,000	1,035,000
" " J‡	1,802,000	1,659,000	1,705,000
" " H‡	1.841.000	1.616.000	1.710.000

* Determined by Dr. Oscar Gawron at the International Vitamin Corporation.

† Saponified according to the procedure of Oser, Melnick, and Pader (13).

‡ Unsaponifiable fraction obtained by the commercial saponification process of the International Vitamin Corporation (14).

of the color with vitamin A) are less than one-third of those at 15 minutes. Thus the interference of vitamin D and related sterols upon the GDH reaction is negligible.

The stability of the violet color produced by vitamin A and GDH suggested the possibility of employing the visual method in the estimation of the vitamin. Such a method would be useful in the field or in those laboratories where a spectrophotometer is not available. Solutions containing 1.26, 2.14, 3.54, and 4.04 I.U. of vitamin A in 1 ml. of reaction mixture were compared against a standard of 2.64 I.U. of vitamin A with the micro cups of the Bausch and Lomb visual colorimeter. The values calculated from the readings were 1.06, 2.12, 3.18, and 4.44 I.U. of vitamin A, respectively.

In many natural products such as food and blood, carotene is often found with vitamin A and interferes in its colorimetric estimation. At present the most widely used method of evaluating this interference is that of Dann and Evelyn (9) which consists in reading the absorption of the yellow solution of carotene and vitamin A at 440 mμ before the addition of antimony trichloride, thereby determining the amount of carotene present from a calibration curve, and subtracting from the maximum absorption obtained with antimony trichloride the absorption that the amount of carotene found would give with the reagent.

Another method has recently been proposed by Urban, Milder, and Carruthers (16) by which vitamin A and carotene are determined mutually and independently by means of a special photoelectric colorimeter that splits the beam of light emerging from a solution of vitamin A and antimony trichloride into two, one passing through a 589 mμ filter measuring β-carotene at 0°, and the other passing through a 620 mμ filter measuring vitamin A. No data are given to substantiate the claim.

The reaction of carotene with GDH was investigated under the same conditions by which the violet color with vitamin A was obtained. Upon the addition of the reagent to a mixture of α- and β-carotene (1:9) in chloroform, a green color appears within 2 minutes. Fig. 5 shows the absorption of the color at the end of 6 minutes. Two maxima were observed, one at 475 mμ and a second at 625 mμ, as well as one minimum at 550 mμ. This minimum coincides with the maximum for vitamin A, while the other maxima are almost at the minima for the vitamin.

Fig. 3 shows the relationship between the absorption of the green color at 550 mμ and time. It is constant at from 6 to 15 minutes.

The relationship between the concentration of carotene and light absorption at 550 mμ obeys Beer's law. This is shown in Fig. 6. This wavelength was chosen in order that we might be able to correct for the presence of carotene in the determination of vitamin A.

The next study made was a comparison of the interference of carotene upon the reaction of vitamin A with GDH and with antimony trichloride. 1 ml. of chloroform containing 18.53 I.U. of vitamin A and known increments of carotene was used in this study. The results are presented in Table II. 6 minutes were chosen in the GDH reaction because at that time both the vitamin A and carotene colors are stable (see Fig. 3). From the last column of Table II, it can be seen that in the antimony trichloride reaction the color due to 1 γ of carotene is equivalent to about 0.24 I.U. of vitamin A, whereas in the GDH reaction the color due to the same amount of carotene is equivalent to about 0.49 I.U. of vitamin A.

It seemed likely (see Fig. 3) that the interference of carotene in the GDH reaction would have been much less had a shorter period of time been

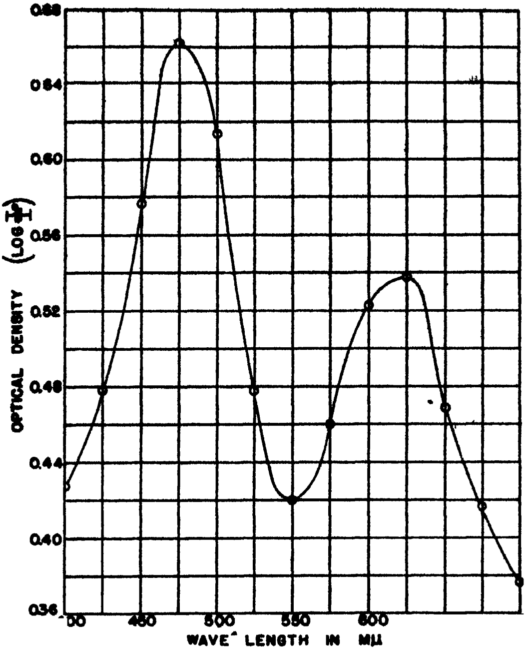


FIG. 5. Absorption curve of the green color produced by carotene (90 per cent of β- and 10 per cent of α-) and glycerol 1,3-dichlorohydrin. The solution contained 122.6 γ in 5.0 ml. of solution.

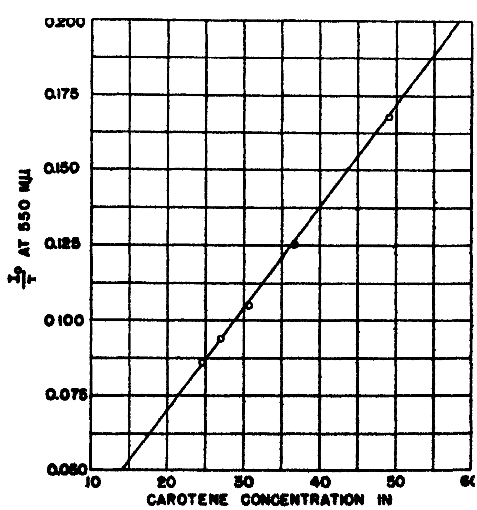


FIG. 6. Relationship between absorption at 550 mμ and the carotene (90 per cent β- and 10 per cent of α-) concentration.

taken before the maximum absorption of the solution was read. Therefore, the interference of carotene upon the immediate blue color produced by the action of GDH on vitamin A was studied. Examination of Table II shows that in this reaction the color due to 1 γ of carotene is equal to about 0.17 I.U. of vitamin A.

The above studies suggest two methods of determining vitamin A (in solutions containing carotene) after the amount of carotene present is evaluated by the method of Dann and Evelyn (9) as described above. One method is to measure the absorption of the solution with GDH at 550 mμ at the end of 6 minutes. Subtract from the absorption obtained

TABLE II

Interference of Carotene in Colorimetric Determination of Vitamin A (18.5 I.U.)

Reagents and conditions	Carotene	Density, $\log \frac{I_0}{I}$	Increment in density due to carotene	Increment as vitamin A per 1 γ carotene
	γ			*I.U.*
Glycerol 1,3-dichlorohydrin		0.146		
550 mμ	13.4	0.204	0.058	0.547
Absorption, 6 min.	20.0	0.222	0.076	0.481
After mixing	26.7	0.244	0.098	0.465
	33.4	0.264	0.118	0.448
$SbCl_3$		0.678		
615 mμ	13.4	0.796	0.118	0.238
Maximum absorption of immediate color	20.0	0.854	0.176	0.238
	26.7	0.914	0.236	0.239
	33.4	0.963	0.285	0.231
Glycerol 1,3-dichlorohydrin		0.195		
625 mμ	13.4	0.216	0.021	0.149
Maximum absorption of immediate color	20.0	0.231	0.036	0.170
	26.7	0.243	0.048	0.170
	33.4	0.254	0.059	0.168

the increment due to carotene. This is read from a calibration chart previously prepared for various amounts of vitamin A and carotene by the method shown in Table II. This procedure possesses the advantages pointed out in this paper for the GDH reaction. Its disadvantage compared with the antimony trichloride method lies in the greater interference of carotene and in the need of an empirical correction chart.

A second method of evaluating carotene interference is to measure the absorption of the solution with GDH at the end of 5 seconds at 625 mμ. Subtract the increment due to carotene as described above. This procedure also possesses the advantages of the GDH reaction, except that the color must be read immediately owing to its rapid change to the violet

color. The carotene interference is about 30 per cent less than it is in the antimony trichloride reaction.

A more detailed study of the interference of carotene is now being undertaken and will be presented in a paper dealing with the application of the GDH reaction in the estimation of vitamin A in blood.

EXPERIMENTAL

Apparatus—All determinations were made in a Coleman universal spectrophotometer, model 11, with Filter PC-4 for the region of 400 to 700 mμ. This instrument employs a monochromatic band of light 35 mμ wide (which can be located with a precision of less than 2.0 mμ). For this reason, $L^{1\%}_{1\,\text{cm.}}$ values are reported instead of $E^{1\%}_{1\,\text{cm.}}$ values, which are used for pure monochromatic light. Direct readings of per cent transmission were made on the galvanometer scale by setting the blank at 100. The absorption path was 1.3 cm.

Reagents—

Practical glycerol 1,3-dichlorohydrin from the Eastman Kodak Company was stored in the ice box and brought to 25° before use. Each new batch should be standardized with known solutions of vitamin A.

GDH obtained from other firms possessed little or no chromogenic property with vitamin A. However, it was found that by distilling the inactive reagent, at 30 to 40 mm. pressure, in the presence of 1 to 2 per cent antimony trichloride a product was obtained possessing properties similar to the practical GDH from the Eastman Kodak Company.

Chloroform, analytical reagent grade, dried over anhydrous sodium sulfate, distilled, and kept over the same drying agent.

Antimony trichloride (reagent grade). A saturated solution in chloroform was prepared by shaking 90 gm. of the trichloride (from an unopened bottle) with 300 ml. of chloroform at room temperature. The saturated solution was filtered before using.

The *standard vitamin A solution* was prepared by dissolving a weighed amount of a distilled vitamin A concentrate from Distillation Products, Inc., Rochester, New York, (potency 500,000 I.U. per gm.) in a known volume of chloroform. This standard remained stable for considerable periods of time as shown by the constant $L^{1\%}_{1\,\text{cm.}}$ values it gave with the GDH and antimony trichloride reagents with which it was frequently checked.

The conversion factor for converting international units of the concentrate into micrograms of vitamin A was 0.292. This was obtained by measuring the extinction coefficient of the concentrate in isopropanol at 328 mμ with a Beckman spectrophotometer. From this value, and by taking the $E^{1\%}_{1\,\text{cm.}}$ equal to 1800 (calculated from the data presented in a

paper by Oser *et al.* (13) who have also shown that the $E^{1\%}_{1\,\text{cm.}}$ of distilled vitamin A esters is the same at 328 mμ as the $E^{1\%}_{1\,\text{cm.}}$ of the saponified esters at 325 mμ), the weight of vitamin A per gm. of concentrate was calculated. It is noteworthy that using this conversion factor we obtained almost the same $E^{1\%}_{1\,\text{cm.}}$ that Baxter and Robeson (12) did for crystalline vitamin A alcohol. All such conversion factors are in doubt at present until proved by exact multiple level bioassays from different laboratories.

The *standard carotene solution* was prepared by dissolving a weighed amount of carotene (90 per cent of β- and 10 per cent of α-) from the S. M. A. Corporation in a known volume of chloroform.

Color Development

Temperature—All colors were developed in a constant temperature bath at 25°, and all reagents were preheated to this temperature.

Use of Practical Glycerol 1,3-Dichlorohydrin—1.0 ml. of chloroform containing vitamin A, carotene, or a mixture of both was pipetted into a 10 ml. glass-stoppered graduate. 4 ml. of reagent were added with a bulb pipette, and the graduate stoppered and inverted several times to insure homogeneous mixing. It was then placed in a 25° water bath, and at the end of the desired incubation period (we recommend 2 minutes for the determination of vitamin A), the solution was poured into a cuvette and its maximum absorption read.

To read the maximum absorption of the immediate blue color, the above procedure was revised as follows: The chloroformic solution containing vitamin A was measured with a pipette directly into the cuvette. Then, with the cuvette held in one hand, 4.0 ml. of the reagent were added in a rapid stream from a fast delivery pipette. The cuvette was vigorously tapped with the forefinger of the other hand several times, placed in the cuvette carrier, and the maximum absorption of the blue color was read. The time elapsed from the addition of the reagent to the reading of the maximum absorption should not exceed 5 seconds.

Use of Antimony Trichloride Reagent—To 1.0 ml. of chloroform containing the vitamin A, which was measured with a pipette into a cuvette, were added 3.0 ml. of antimony trichloride reagent with a fast delivery pipette. The maximum absorption was immediately read.

Visual Colorimetric Technique—The determination was carried out at room temperature with the violet color produced by the reaction of GDH with vitamin A. With each unknown a standard solution of vitamin A was run, the violet color in both being simultaneously developed. The unknowns (prepared from a known standard) were read against the standard at any time from 2 to 10 minutes after the reagents were mixed.

All the densities reported in this paper are the averages of duplicate determinations.

SUMMARY

A new colorimetric reagent, glycerol 1,3-dichlorohydrin, is proposed for estimating vitamin A. It possesses the advantage over the antimony trichloride reagent that the color developed with vitamin A is stable for from 2 to 10 minutes, which permits its absorption to be determined with ease. Other advantages are that the use of the reagent requires no special precautions and possesses good stability.

Spectrophotometric data are presented of the immediate transient blue color and the stable secondary violet color produced by reaction of the reagent with vitamin A, as well as the color formed by its reaction with carotene.

The interference of carotene upon the determination of vitamin A with the reagent has been studied.

The authors are indebted to Dr. Oscar Gawron, Research Laboratory, International Vitamin Division, American Home Products Corporation, New York, for the samples of fish liver oils which were used in this investigation.

BIBLIOGRAPHY

1. Heilbron, I. M., Jones, W. E., and Bacharach, A. L., in Harris, R. S., and Thimann, K. V., Vitamins and hormones, New York, **2**, 189–194 (1944).
2. Loofbourow, J. R., in Harris, R. S., and Thimann, K. V., Vitamins and hormones, New York, **1**, 132 (1943).
3. Morton, R. A., Application of absorption spectra to the study of vitamins, hormones, and coenzymes, London, 2nd edition, 84–102 (1942).
4. Hickman, K., Annual review of biochemistry, Stanford University, **12**, 364–367 (1943).
5. Wilkie, J. B., *Ind. and Eng. Chem., Anal. Ed.*, **13**, 209 (1941).
6. Rosenberg, H. R., Chemistry and physiology of the vitamins, New York, 78–82 (1942).
7. Oser, B. L., Melnick, D., and Pader, M., *Ind. and Eng. Chem., Anal. Ed.*, **15**, 724 (1943).
8. Oser, B. L., Melnick, D., Pader, M., Roth, R., and Oser, M., Abstracts, Division of Agricultural and Food Chemistry, American Chemical Society, 108th meeting, New York, 1 (1944).
9. Dann, W. J., and Evelyn, K. A., *Biochem. J.*, **32**, 1008 (1938).
10. Kimble, M. S., *J. Lab. and Clin. Med.*, **24**, 1055 (1939).
11. Koehn, C. J., and Sherman, W. C., *J. Biol. Chem.*, **132**, 527 (1940).
12. Baxter, J. G., and Robeson, C. D., *J. Am. Chem. Soc.*, **64**, 2411 (1942).
13. Oser, B. L., Melnick, D., and Pader, M., *Ind. and Eng. Chem., Anal. Ed.*, **15**, 717 (1943).
14. Marcus, J. K., *J. Biol. Chem.*, **80**, 9 (1928).
15. Sobel, A. E., Mayer, M., and Kramer, B., *Ind. and Eng. Chem., Anal. Ed.*, **17**, 160 (1945).
16. Urban, F., Milder, B., and Carruthers, C., *Biochem. J.*, **37**, 295 (1943).

NOTE ON THE REPORTED FORMATION OF UREA FROM GLUTAMINE BY LIVER EXTRACTS, AND ON THE PREPARATION OF GLUTAMINE FREE FROM ARGININE

By REGINALD M. ARCHIBALD

(*From the Hospital of The Rockefeller Institute for Medical Research, New York*)

(Received for publication, April 9, 1945)

The writer recently reported (1) that, when cell-free liver extract acts on glutamine, urea, equivalent to about 3 per cent of the glutamine amide N, is formed. Since that time it has been found that the preparation of beet glutamine used was contaminated with exactly the amount of arginine required to yield the small amount of urea formed. The arginine present would be converted into urea by the arginase that is always present in mammalian liver. With glutamine solutions freed from arginine the formation of urea reported in the previous paper (1) does not occur. The author therefore wishes to withdraw the statement that he has observed formation of small amounts of urea on incubation of glutamine with liver extract.

The glutamine preparation previously employed proved to be 94 per cent pure when analyzed by the glutaminase method (2). The preparation had been tested for arginine by Dubnoff's modification (3) of the colorimetric Sakaguchi reaction and gave negative results, but we have since found that glutamine interferes with the Sakaguchi method for arginine. If the Sakaguchi reagent is added to a solution containing both arginine and glutamine, the increase in optical density (525 mμ wave-length) is less than if only the arginine were present. The interference is due to the sum of two effects of the glutamine; it diminishes the optical density of the Sakaguchi reagent solution itself and decreases the color formed by the reaction with arginine.

Recrystallization from water failed to free the glutamine from contaminating arginine. However, we have been able to prepare glutamine solutions free of arginine by the following adsorption procedure, and to measure the arginine in the eluate of the adsorbed material.

The lower 10 cm. of a 30 cm. length of glass tubing (6 mm. internal diameter) were filled with a column of Decalso. The Decalso was washed with 20 cc. of a 10 per cent solution of NaCl, then with water (about 50 cc.) until the filtrate was chloride-free, and was dried by drawing air through the column; 25 cc. of a 1 per cent solution of the arginine-containing glutamine were then passed through the cylinder. The filtrate contained no arginine, but contained over 90 per cent of the glutamine.

To regain the adsorbed arginine for determination, the Decalso column was freed as completely as possible of adherent glutamine solution by

suction, and was then washed by passing 20 cc. of water (Eluate I) through it, followed by 25 cc. of a 3.0 per cent solution of NaCl (Eluate II).

The arginine in the two eluates was measured by hydrolyzing with arginase (liver extract), and determining the resulting urea colorimetrically (4). Eluate I contained 25 per cent of the adsorbed arginine and all of the glutamine previously left in the column.[1] Eluate II contained the remaining 75 per cent of the arginine, and no glutamine.

The nitrogen of the total urea formed by the action of arginase on the two eluates was equivalent to 2.1 per cent of the theoretical amide nitrogen of one glutamine preparation, and indicated 1.3 per cent of arginine present in the preparation. With another glutamine preparation the figures were 3.2 and 2.0 respectively. When arginine in these two preparations was determined directly with arginase, the amounts found were identical with the sum found in the eluates.

The Sakaguchi reaction failed to detect the arginine present in Eluate I, because enough glutamine was present to inhibit the development of color by the arginine. In Eluate II, however, the Sakaguchi reaction indicated the same amount of arginine revealed by analysis with arginase.

Glutamine in the two eluates was determined by heating 1 cc. portions of each eluate to 100° for 4 hours with 0.5 cc. portions of 0.5 M potassium phosphate buffer of pH 6.0. Each solution was made up to 20 cc. with water, and 1 cc. of Nessler's reagent was added. The ammonia formed by hydrolysis of the amide group of the glutamine was then measured colorimetrically. Eluate I contained 6 per cent of the original glutamine; Eluate II contained none.

Canavanine forms urea when treated with liver arginase, and hence the possibility was considered that canavanine rather than arginine was the contaminant of the glutamine previously used. However, both Eluate I and Eluate II gave negative tests for canavanine by a colorimetric method mentioned in a previous report (5), and the liberation of urea from the eluate by the action of arginase was completed much more rapidly than if canavanine were the substrate. Hence it appears that the contaminant was arginine rather than canavanine.

BIBLIOGRAPHY

1. Archibald, R. M., *J. Biol. Chem.*, **157**, 519 (1945).
2. Archibald, R. M., *J. Biol. Chem.*, **154**, 643, 657 (1944).
3. Dubnoff, J. W., *J. Biol. Chem.*, **141**, 711 (1941).
4. Archibald, R. M., *J. Biol. Chem.*, **157**, 507 (1945).
5. Archibald, R. M., and Hamilton, P. B., *J. Biol. Chem.*, **150**, 155 (1943).

[1] When arginine alone is present in the column, washing with water removes no arginine, but, when glutamine is also present, all of the glutamine and about 25 per cent of the arginine are eluted with water. Likewise a 0.3 per cent solution of NaCl used as a wash, as recommended by Dubnoff (3), elutes no arginine when glutamine is absent, but elutes part of the arginine when glutamine is present.

A STUDY OF THE INTERMEDIATES OF ACETATE AND ACETO-ACETATE OXIDATION WITH ISOTOPIC CARBON

By JOHN M. BUCHANAN, WARWICK SAKAMI, SAMUEL GURIN, AND D. WRIGHT WILSON

(*From the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia*)

(Received for publication, May 17, 1945)

The identity of the intermediates of acetate and acetoacetate oxidation *in vivo* has until recently remained relatively obscure. The first suggestion concerning the mode of metabolism of these compounds came from the experiments of Quastel and Wheatley (1). By virtue of their finding that malonate, a poison of the succinoxidase system, inhibits fatty acid and ketone body oxidation in tissue slices, Quastel and Wheatley suggested that the oxidation of these substances was related to reactions of the tricarboxylic acid cycle. In confirming this observation, Edson and Leloir (2) extended this hypothesis with the further postulation that pyruvate and acetate condense, forming α-ketoglutarate. More direct evidence for the conversion of acetoacetate into members of the tricarboxylic acid cycle has been provided by Breusch (3) and by Wieland and Rosenthal (4), who have independently reported that extracts or breis of kidney or muscle tissue are capable of forming more citrate from oxalacetate plus acetoacetate (or acetate) than from oxalacetate alone. Breusch has claimed that extracts of muscle convert not only acetoacetate but also other homologous β-keto acids to citric acid. Wieland and Rosenthal have isolated and characterized the citric acid formed by the reaction of oxalacetate with acetoacetate in kidney breis and have postulated a direct condensation of the two substances.

The interpretations which these investigators have placed upon their experiments have been criticized by Weil-Malherbe and by Krebs and Eggleston. Weil-Malherbe (5) was unable to observe significant effects of members of the tricarboxylic acid cycle on the oxygen uptake, formation of citric acid, and disappearance of ketone bodies in slices of rat brain and kidney, either aerobically or anaerobically. Krebs and Eggleston (6), however, using sheep heart and sheep kidney, confirmed the observation that acetoacetic acid can increase the yields of citric acid (and its breakdown products) in the presence of oxalacetic acid. In both tissues, the removal of acetoacetic acid was stimulated by members of the tricarboxylic acid cycle. The effect was greater under anaerobic than under aerobic conditions. There was one exception, however. Oxalacetate did not

stimulate acetoacetate disappearance aerobically. From a complete analysis of the products of the anaerobic interaction between acetoacetate and oxalacetate, these investigators came to the conclusion that the extra acetoacetic acid disappearing by interaction with oxalacetic acid could be quantitatively recovered as β-hydroxybutyric acid, and that the reciprocal effect of acetoacetate and oxalacetate on the metabolism of the other was indirect rather than direct. They believe that, by acting as a hydrogen acceptor, acetoacetate stimulates the oxidative conversion of oxalacetate to citric acid.

In the experiments reported herein, acetate and acetoacetate tagged with C^{13} (*i.e.*, $CH_3 \cdot C^{13}OOH$ and $CH_3 \cdot C^{13}O \cdot CH_2 \cdot C^{13}OOH$), were used to study the intermediates of the oxidation of these substances in homogenates of guinea pig kidney. It was found that under the conditions of our experiments the organic acids of the tricarboxylic acid cycle are a major pathway in these oxidations.[1]

EXPERIMENTAL

Organic Synthesis[2]—Carboxyl-tagged acetate was synthesized from $C^{13}O_2$ by the Grignard reaction. Ethyl acetate, prepared by heating anhydrous sodium acetate with diethyl sulfate, was converted into acetoacetate by the method of Hudson, Dick, and Hauser (8).

Tissue Reactions—The aerobic disappearance of acetoacetate in homogenates of guinea pig kidney is stimulated approximately 3-fold by the addition of any one of the organic acids of the tricarboxylic acid cycle. Homogenates of guinea pig kidney metabolize acetoacetate at a rate ($Q_{\text{acetoacetate}} = -1.5$) lower than that previously reported for slices of guinea pig kidney ($Q = -4.0$) (1). The addition of any of the organic acids of the tricarboxylic acid cycle to make concentrations of 0.003 M in homogenates raises the rate of disappearance of acetoacetate to that in slices.

Since, in the present experiments involving the use of C^{13} it was necessary to add a sufficient amount of dicarboxylic acid so that either it or an immediate product of its metabolism could be conveniently isolated, the effect of these organic acids in higher concentrations was studied. Increasing the concentration of α-ketoglutarate, succinate, fumarate, and malate from 0.003 to 0.03 M did not materially affect the rate of acetoacetate disappearance. Oxalacetate at this latter concentration, however, depressed acetoacetate disappearance considerably.

In the following series of experiments sodium α-ketoglutarate or sodium succinate was added in concentrations of 0.015 M. In a typical experi-

[1] A preliminary note has been published in this *Journal* (7).

[2] A detailed description of the methods for the synthesis of acetoacetate and acetate in small quantities will be presented in a separate communication.

ment 10 gm. of guinea pig kidney were homogenized in a Potter-Elvehjem homogenizer in approximately 65 ml. of Krebs' phosphate-saline solution. This homogenate was added to 25 ml. of a solution containing sodium bicarbonate, the sodium salt of a dicarboxylic acid, and isotopic sodium acetoacetate. The final concentrations of these substances were 40, 15, and 4 mM per liter respectively. The solution was shaken vigorously at 37° for 40 to 60 minutes, during which time a gaseous mixture of 95 per cent O_2-5 per cent CO_2 was bubbled through to insure the adequate removal of isotopic CO_2 produced by the metabolism of the tissue. By this means the isotopic content of the bicarbonate of the solution was kept at a very low level. At intervals during the incubation, aliquots were removed and acidified with a buffer solution (pH 4), the CO_2 evolved being trapped in barium hydroxide for isotopic analysis. In all, four different types of experiments have been carried out. These are summarized in Table I.

Isolation of Compounds—Certain acids of the tricarboxylic acid cycle thought to be formed by the metabolism of either isotopic acetate or acetoacetate were isolated and carefully purified. The *p*-nitrobenzylthiuronium salts were prepared (9) whenever possible because of the ease with which they can be split by dilute H_2SO_4 into *p*-nitrobenzylthiuronium sulfate and the free organic acid. The latter was extracted with ether and precipitated from aqueous solution as a heavy metal salt. Isotopic analyses were usually made on these inorganic salts, thus avoiding the dilution of the isotope by non-isotopic carbon which would occur if isotopic analyses were made on organic derivatives. The isolation and purification of each of the organic acids studied are described below.

Acetoacetic Acid—The isotopic content of the acetoacetic acid used was determined by conversion into CO_2 and acetone. The former substance was trapped in $Ba(OH)_2$ and acetone was precipitated by the Denigès reagent. From the isotopic analysis of either of these separate fractions, the isotopic content of the entire acetoacetate molecule was calculated.

Fumaric Acid—At the conclusion of the incubation period in Experiments 1A and B, Table I, about 10 ml. of 15 per cent metaphosphoric acid were added to the 100 ml. of solution and the protein precipitate centrifuged off. The supernatant was concentrated *in vacuo* to about 10 ml. and was then extracted with three 25 ml. portions of ether. The ether was evaporated and the residue taken up in water. Fumaric acid was precipitated as the mercurous salt, which was recrystallized from 5 per cent HNO_3 as described by Stotz (10), and then decomposed by H_2S. The solution of the free acid was neutralized, evaporated to a small volume, and treated with *p*-nitrobenzylthiuronium chloride. The product, after several recrystallizations from hot 70 per cent ethanol, melted at 157° and gave no depression upon mixing with an authentic sample. The pure

di-*p*-nitrobenzylthiuronium salt was dissolved in dilute sulfuric acid and the solution extracted with ether. After removal of the ether, the fumaric acid was again precipitated with mercurous nitrate in 5 per cent HNO_3. This mercurous fumarate was oxidized with the Van Slyke mixture (11), the evolved CO_2 trapped in liquid air, and a sample of the CO_2 taken for determination of its C^{13} concentration in the mass spectrometer.

Succinic Acid—At the conclusion of incubation in Experiment 4, protein was precipitated and the succinic acid, which had resulted from the oxidation of α-ketoglutaric acid, was extracted from the concentrated supernatant with ether. An attempt was made to isolate α-ketoglutaric acid from an alkaline aqueous extract of the ether solution, but no precipitate resulted after the addition of semicarbazide hydrochloride. Subsequent analysis demonstrated that most of the α-ketoglutarate had disappeared during the experimental period. This solution, containing organic acids together with semicarbazide, was acidified with H_2SO_4 and extracted with ether in the Kutscher-Steudel apparatus. The ethereal extract was taken up in 5 per cent HNO_3, and $HgNO_3$ added. A small precipitate was obtained, but most of the material was not precipitated by this reagent. The mercurous compound (probably impure mercurous fumarate) was centrifuged off and the filtrate was neutralized with NaOH. Addition of excess alkali precipitated the mercurous ion as Hg_2O, which was centrifuged off. The supernatant was acidified to pH 2 with HNO_3, 2 ml. of 20 per cent $AgNO_3$ were added, and the solution was brought to pH 5 with 0.5 N NH_4OH. A copious precipitate of silver succinate was obtained. Succinic acid was purified by acidification and reprecipitation of the silver salt at pH 5. A solution of the salt was acidified with H_2SO_4 and continuously extracted with ether. The succinic acid thus extracted was dissolved in a very small volume of water, neutralized with NaOH, and precipitated as the di-*p*-nitrobenzylthiuronium salt. After several recrystallizations from hot absolute ethanol, the thiuronium salt melted at 150° and gave no depression upon admixture with an authentic preparation. It was dissolved in excess H_2SO_4 and the succinic acid extracted with ether. The recovered succinic acid was then converted into the silver salt, oxidized as such, and analyzed for C^{13}.

α-Ketoglutaric Acid—In Experiment 2, α-ketoglutaric acid was added to stimulate acetoacetate oxidation. Protein was precipitated with sodium tungstate and an excess of H_2SO_4. The supernatant, concentrated under reduced pressure, was extracted for 5 hours in the Kutscher-Steudel apparatus. Under our conditions, complete extraction of α-ketoglutaric acid along with the other dicarboxylic acids (except malate) was effected in this time. The ether was evaporated and the residue taken up in 1 ml. of water. The α-ketoglutaric acid was precipitated as the semicarbazone

and recrystallized from water three times. It decomposed at 199.0° (uncorrected), as did an authentic sample of α-ketoglutaric acid semicarbazone and a mixture of the two specimens.[3]

Degradation of Organic Acids—Since the position of the isotope present in the organic acids isolated was of theoretical interest, fumaric acid and α-ketoglutaric acid semicarbazone were degraded by controlled oxidation.

Fumaric Acid—Fumaric acid was oxidized with acid permanganate, each mole yielding 1 mole of formic acid and 3 moles of CO_2 (14). The formic acid originates from a methine carbon of fumaric acid and the carbon dioxide from the carboxyl carbons and one of the methine carbons[4] (15).

α-Ketoglutaric Acid Semicarbazone—α-Ketoglutaric acid semicarbazone was oxidized quantitatively[5] by acid permanganate, according to the following equation:

$$\begin{array}{c} NH_2\cdot CO\cdot NH\cdot N \\ \| \\ HOOC\cdot CH_2\cdot CH_2\cdot C\cdot COOH + 3O \longrightarrow \end{array} \qquad (1)$$

$$HOOC\cdot CH_2\cdot CH_2\cdot COOH + 2CO_2 + N_2 + NH_3$$

In this reaction one of the molecules of carbon dioxide is derived from the carbon of the semicarbazide group and the other from the carboxyl carbon α to this group. The resulting succinic acid was extracted from the oxidation mixture and precipitated as the silver salt at pH 5. In the following discussion, these two oxidation products of α-ketoglutaric acid semicarbazone are referred to as the "CO_2 fraction" and the "succinic acid fraction."

[3] The decomposition point, 199°, is considerably lower than the melting point, 220°, of the semicarbazone of α-ketoglutaric acid reported by Blaise and Gault (12) and is probably a stereoisomeric modification. The identity of the semicarbazone prepared from an authentic sample of α-ketoglutaric acid was established by an acidimetric determination of molecular weight (found 204, theory 203), and by a determination of the semicarbazone by the method of Veibel (13) (moles of ammonia per mole of semicarbazone, found 1.01, theory 1.00).

[4] 10.5 ml. of 0.1 N H_2SO_4, 1.75 ml. of 1 N $KMnO_4$, and 1.75 ml. of water were mixed and aerated rapidly for 10 minutes to remove CO_2 formed from impurities. 21 mg. of fumaric acid in 7 ml. of water were added, and the portions of CO_2 liberated during the first 20 minutes and during the next 2 hours were collected separately in barium hydroxide. With the strength of acid used, the initial oxidation is very rapid but is followed by a gradual oxidation of the formic acid produced.

[5] 0.3 ml. of 1.5 N $KMnO_4$ was added to 1 ml. of 4 N H_2SO_4 and the solution aerated to remove any CO_2 produced by the oxidation of impurities. 7 mg. of α-ketoglutaric acid semicarbazone were introduced in 4 ml. of water, followed by 0.5 ml. of water. The resulting CO_2 was collected *in vacuo* in a liquid air trap.

Results

The results of five experiments are presented in Table I. All of the intermediates of the tricarboxylic acid cycle isolated after incubating homogenates of guinea pig kidney with isotopic acetate or acetoacetate were found to contain a significant excess of C^{13}. This proves conclusively that some of the acetate and acetoacetate is metabolized by a pathway which, in part, is identical with the aerobic metabolism of carbohydrate. In Column 3, Table I, are reported the amounts of acetic acid or acetoacetic acid metabolized by 10 gm. (wet weight) of homogenized kidney in 100 ml.

TABLE I

Metabolism of Acetate and Acetoacetate

Experiment No.	Isotopic material added	Amount of acetoacetate or acetate metabolized	Non-isotopic organic acid added	Dicarboxylic acid isolated	C^{13} concentration, atoms per cent excess: Initial acetoacetate or acetate	Dicarboxylic acid isolated	$NaHCO_3$ (average value)
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
		mM					
1A*	Acetoacetate	0.25	Succinic	Fumaric	3.80	0.22	0.12
1B*	"	0.34	"	"	3.98	0.30	0.01
2	"	0.32	α-Ketoglutaric	α-Ketoglutaric	3.98	0.50‡	0.03
3*	CO_2	0.10	Succinic Acetoacetic	Fumaric	0.00	0.00	2.28
4†	Acetate	0.31	α-Ketoglutaric	Succinic	3.88	0.28	0.01

* 60 minute experiment.

† 40 minute experiment.

‡ This value calculated from the observed C^{13} content of α-ketoglutaric acid semicarbazone.

of solution. These acids were determined by the methods of Friedemann (16) and Edson (17) respectively. In all but Experiment 3, the concentration of C^{13} in the bicarbonate was maintained at a low level by continuous bubbling of a non-isotopic gas mixture (95 per cent O_2-5 per cent CO_2) through the solution. Average values for the isotopic content of the bicarbonate of the solution are included in Column 8, Table I.

In Experiment 3, non-isotopic acetoacetate was incubated with non-isotopic sodium succinate in a medium containing bicarbonate labeled with C^{13}. The pure fumaric acid isolated at the conclusion of the experiment contained a normal concentration of C^{13}, indicating that under the conditions of our experiments CO_2 is not incorporated into these substances.

In Table II are reported results of experiments in which fumaric acid and α-ketoglutaric acid semicarbazone were partially degraded by oxidation. The carbon dioxide obtained by the oxidation of the methine carbons of fumaric acid ("formic acid" fraction in Table II) contained no excess of C^{13}, proving that all of the excess C^{13} of the isotopic fumaric acid was in the carboxyl carbons. Since the methine carbons of fumaric acid originated directly from the methylene carbons of succinic acid and indirectly from the methylene carbons of α-ketoglutaric acid, it is concluded that

TABLE II

Concentration of Isotope in Fractions Obtained by Degrading Metabolic Products of Acetoacetic Acid

Substance	Origin of fraction	C^{13} concentration, atom per cent excess	
		Experimental	Calculated
(1)	(2)	(3)	(4)
Fumaric acid	Experiment 1B	0.30	
"CO_2" fraction	2 carboxyl carbons + 1 methine carbon	0.43*	
"Formic acid" fraction	1 methine carbon	0.00	
1 carboxyl carbon			1.20
α-Ketoglutaric acid semicarbazone	Experiment 2	0.42	
"CO_2" fraction	C of semicarbazide and C of α-carboxyl	0.12	
"Succinic acid" fraction	Remaining 4 carbons of α-ketoglutaric acid semicarbazone	0.59	
α-Carboxyl carbon			0.24
γ-Carboxyl "			2.36

* In the oxidation of fumaric acid with acid permanganate 6.8 mg. of isotopic fumaric acid were diluted with 14 mg. of non-isotopic fumaric acid. The isotope excess obtained (0.14) has been corrected for the dilution with non-isotopic fumaric acid as follows: $(0.14 \times 20.8)/6.8 = 0.43$.

none of these carbon atoms contained excess isotope. Furthermore, in view of present theories on the mechanism of formation of α-ketoglutaric acid from oxalacetate and acetoacetate, the assumption is made that the carbon atom of the keto group of α-ketoglutaric acid isolated in these experiments is non-isotopic. Since one of the carboxyl carbons of either succinic or fumaric acid is derived from this carbon atom, it is therefore non-isotopic. For this reason the assumption is made in the calculations in Column 4, Table II, that all of the isotope of the recovered succinic and fumaric acids resided in one carboxyl group only and that this carboxyl group was derived from the carboxyl group of α-ketoglutaric acid which was

γ to the keto group. On the basis of this assumption it is possible to estimate that the isotope content of the γ-carboxyl carbon of α-ketoglutaric acid was approximately 10 times that of the α-carboxyl carbon (Column 4, Table II).

DISCUSSION

The distribution of isotopic carbon in α-ketoglutaric acid during its formation in pigeon liver mince from pyruvate and isotopic carbon dioxide led Evans and Slotin (18) and Wood *et al.* (19) to conclude that citric acid is not a direct intermediate in pyruvate metabolism. Wood *et al.* have suggested that *cis*-aconitic acid is the product of the oxidative condensation of pyruvic and oxalacetic acids.

The results reported in this paper dealing with the distribution of isotope in α-ketoglutarate likewise furnish information to identify the intermediates formed in the metabolism of acetoacetate. If citric acid were an intermediate in this metabolism, both carboxyl carbons of the resulting α-ketoglutaric acid should contain equal concentrations of C^{13}. It has been estimated, however, that the carboxyl carbon γ to the keto group of α-ketoglutaric acid contained approximately 10 times more isotope than the other carboxyl carbon. Although in this estimation certain assumptions were made, they are not necessary to establish the validity of the conclusion. Without making any assumptions regarding the distribution of isotope in the succinic acid resulting from the oxidation of α-ketoglutarate, it was possible to show experimentally that the average C^{13} excess of these 4 carbon atoms was 2.5 times greater than that of the 5th carbon atom of α-ketoglutaric acid (*i.e.*, the α-carboxyl carbon). For these reasons the authors suggest that *cis*-aconitate or isocitrate or both may be intermediates in the acetoacetate oxidation, but that citrate is not.

The presence of a relatively small excess of isotope in the carboxyl carbon α to the keto group of α-ketoglutaric acid resulting from the metabolism of isotopic acetoacetic acid may arise in several ways. As has been suggested by us previously (7), it may result from the participation of a small amount of *cis*-aconitic acid in an equilibrium reaction with citric acid. Such an equilibrium reaction would distribute the isotopic carbon equally between two of the three carboxyl groups of *cis*-aconitic acid. A more probable explanation is that carboxyl isotopic oxalacetic acid formed by the reactions in Diagram 1 reenters the cycle, combining with a second molecule of labeled acetoacetate. α-Ketoglutaric acid thus formed should contain C^{13} in the carboxyl group α to the keto position, since it is theoretically derived from one of the carboxyl groups of oxalacetic acid. Since isotopic oxalacetic acid produced by the first series of reactions has been diluted once by the added non-isotopic α-ketoglutaric acid and upon re-

entering the cycle is diluted again, it would be expected that the C^{14} concentration of the carboxyl carbon α to the keto group of α-ketoglutaric acid would be relatively small when compared with the concentration in the other carboxyl carbon of this compound.

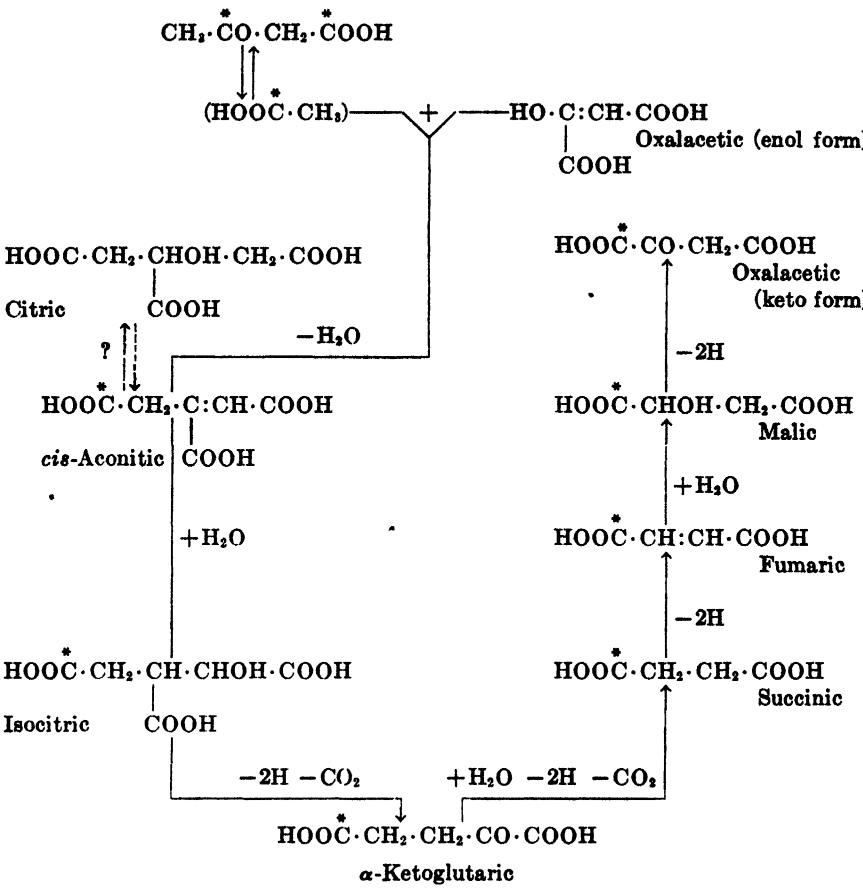


DIAGRAM 1. Proposed mechanism of oxidation of acetoacetate and acetate via the tricarboxylic acid cycle. Although isotopic carbon has been assigned to only one carboxyl of succinic, fumaric, malic, and oxalacetic acids respectively, it is obvious that the isotope may be allocated to one or the other carboxyl (but not both) after formation of the symmetrical succinic acid.

The finding that acetate and acetoacetate as well as pyruvate are oxidized by way of the tricarboxylic acid cycle suggests a correlation of fat and carbohydrate metabolism. Although acetate (20) and acetoacetate (21) have been recognized as products of pyruvate metabolism, they have not been generally accepted as intermediates in the formation of tricar-

boxylic acids from pyruvate. Usually pyruvate and oxalacetate have been considered as the reactants in the condensation, and oxalcitraconic acid has been suggested as a possible product (22). An alternative theory is that pyruvate is first oxidatively decarboxylated into a 2-carbon intermediate, which is condensed with a 4-carbon compound to form a 6-carbon tricarboxylic acid (*cis*-aconitate or isocitrate). Acetoacetate, according to such a theory, would represent a reservoir for this hypothetical 2-carbon intermediate and would be formed in those tissues (*i.e.* liver) in which fat and pyruvate metabolisms are relatively rapid. The 2-carbon intermediate of acetoacetate and pyruvate metabolism might be acetic acid or its phosphorylated derivative, acetyl phosphate. The recent observation by Weinhouse, Medes, and Floyd (23) that a 2-carbon compound is involved in the oxidation of octanoic to acetoacetic acid has initiated a renewed interest in this 2-carbon compound as an important intermediate of fat and ketone body metabolism. Calculations based upon our experimental findings indicate that acetate is oxidized via the organic acids of the tricarboxylic acid cycle at a rate similar to that of acetoacetate. This is further support for the belief that a 2-carbon compound may be an intermediate of acetoacetate oxidation.

A scheme proposing that *cis*-aconitic acid is formed by condensation of a C_2 and a C_4 unit is consistent with existing non-isotopic data concerning the formation or non-formation of carbohydrate from C_3 or C_2 units. Although many 3-carbon chains such as glycerol, pyruvate, lactate, alanine, and propionate may be converted into carbohydrate by the mammalian organism, it has never been conclusively demonstrated that acetic acid or multiples of this C_2 unit (*i.e.* naturally occurring fatty acids) can undergo this same reaction.

At present it is thought that Reaction 2 (Diagram 2) (*i.e.* phosphopyruvic + adenosine diphosphate → pyruvic + adenosine triphosphate) (24) and Reaction 5 (*i.e.* pyruvic + O → carbon dioxide + acetic) are irreversible in the mammalian organism (25). On the basis of experiments with radioactive $C^{11}O_2$, Hastings and his coworkers (26) have postulated that phosphopyruvate may be synthesized from pyruvate by Reactions 3 and 4. According to this mechanism oxalacetate would be an intermediate of glycogen synthesis from pyruvate. Although the conversion of acetic acid into pyruvic acid by reductive carboxylation has not been demonstrated in mammalian tissues, the reverse reaction is a reaction of considerable significance. Bloch and Rittenberg have shown, for instance, that alanine, which may be converted into pyruvic acid, is almost as effective as acetic acid itself in the acetylation of *l*-phenylaminobutyric acid (27).

If the assumption is made that acetic acid is further metabolized by condensing with oxalacetic acid forming *cis*-aconitic or isocitric acid, it is

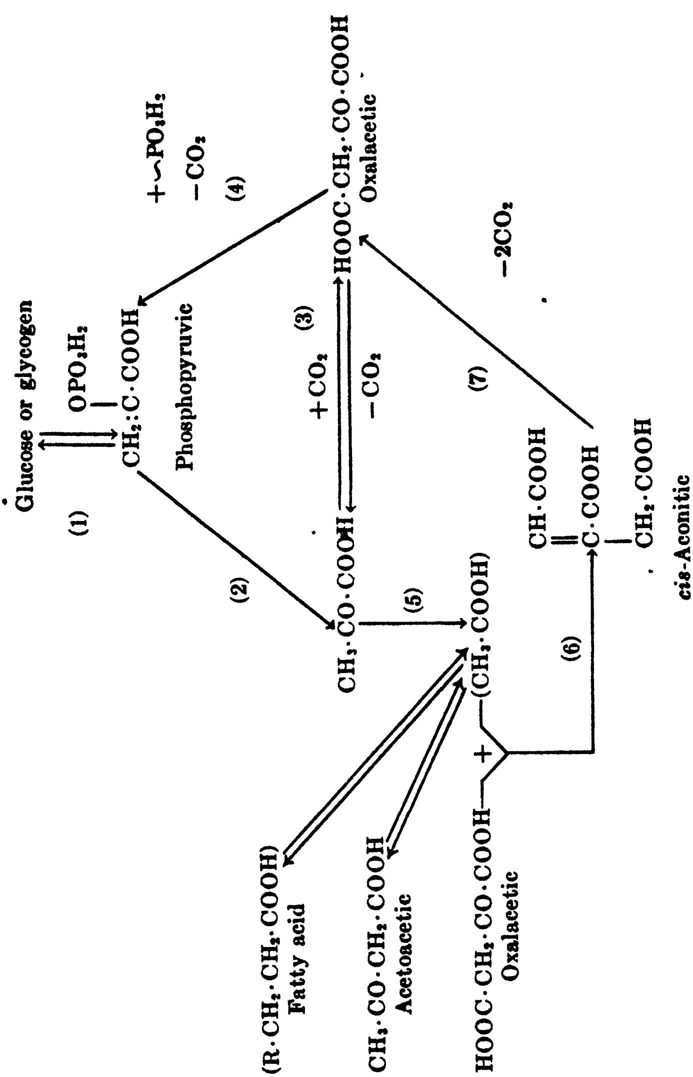


DIAGRAM 2. Integration of carbohydrate and fat metabolism.

possible to explain how acetic acid as well as other fatty acids may be oxidized via a mechanism common to carbohydrate oxidation without effecting a net synthesis of carbohydrate. According to Reactions 6 and 7, Diagram 2, 1 mole of oxalacetic acid reacts with 1 mole of acetic acid to form 2 moles respectively of CO_2 and water and 1 of oxalacetic acid. Thus there has been no accumulation of oxalacetic acid, although 1 mole of acetic acid has been oxidized. The over-all reaction is acetic acid $\rightarrow$ $2CO_2 + 2H_2O$.

The carbons of the acetic acid, however, are not the ones which have formed CO_2. Thus, acetic acid carbon may be found in a carbohydrate precursor (oxalacetate) without causing its accumulation. Such a reaction suggests the need of carbohydrate intermediates for the oxidation of fatty acids. According to this scheme fatty acids and that fraction of pyruvic acid which is converted into acetic acid cannot be converted into carbohydrate. Pyruvic acid, metabolized by the alternative pathway of the Wood and Werkman carboxylation reaction, on the other hand, may be presumed to be a source of carbohydrate. Obviously, these conclusions concerning the metabolism of fatty acids are based upon the assumption that the conversion of acetic acid directly to pyruvic acid does not take place at all or to any appreciable extent in the mammalian organism (28, 29).

The above discussion offers an explanation for the experiments of Buchanan, Hastings, and Nesbett (30), in which carboxyl radioactive butyric acid was administered to white rats along with non-isotopic glucose. Significant amounts of radioactivity were found in the liver glycogen isolated after a 2 hour experimental period. As has been demonstrated by Hastings and his coworkers (31) and by Boxer and Stetten (32), there is a considerable synthesis of glycogen from compounds with carbon chains smaller than glucose after the administration of glucose to fasted rats. Vennesland *et al.* (31) have postulated that phosphorylated derivatives of glucose and the components of the tricarboxylic acid cycle are rapidly brought into equilibrium by enzymatic reactions (Reactions 1 to 4, Diagram 2). According to these equilibrium reactions shown in Diagram 2, it is possible for isotopic carbon of isotopic oxalacetic acid formed during the metabolism of carboxyl radioactive butyric acid to appear in glycogen, even though there has been no increase of either carbohydrate or the carbohydrate precursor, oxalacetic acid.

The inability of these investigators to demonstrate the incorporation of acetate carbon into glycogen *in vivo* under conditions in which butyrate yielded a positive result is at present unexplained. Recently, however, Rittenberg and Bloch (33) have been able to isolate isotopic glutamic and aspartic acids from the carcass and liver of mice and rats fed acetate containing a high concentration of C^{13} in the carboxyl position. The isotope

concentrations in the isolated compounds were small because of the great dilution always encountered in such experiments with intact animals, but were significant.

Since, in our *in vitro* experiments with acetic acid, the dilution factors are known to a certain extent, it is possible to calculate roughly the percentage of metabolized acetic acid which is oxidized via intermediates of the tricarboxylic acid cycle. This may be done when the following are known: (*a*) the initial isotopic concentration of acetate, (*b*) the millimoles of acetate carbon metabolized, (*c*) the millimoles of dicarboxylic acid carbon added to the tissue, and (*d*) the final concentration of isotope in the dicarboxylic acid isolated In the experiment with isotopic acetate, all of the added α-ketoglutarate was metabolized. The main product of metabolism was succinic acid; only small amounts of fumarate (or malate) were found. It was assumed in making the calculation that complete intermixing of the isotopic and non-isotopic compounds occurred and that the concentration of isotope in the succinic acid was equal to the concentration of isotope in the various metabolic products The essential data for this calculation are as follows: (*A*) initial isotopic concentration of acetate, 3.90 atoms per cent excess C^{13}; (*B*) millimoles of acetate carbon metabolized, millimoles of acetate metabolized $\times$ 2 = 0 31 $\times$ 2 = 0.62; (*C*) millimoles of dicarboxylic acid carbon added, 1 5 $\times$ 4 = 6 (only 4 carbons of α-ketoglutaric acid are used in the calculation as the fifth is removed as CO_2); (*D*) final isotopic concentration in the succinic acid isolated, 0.28 atom per cent excess C^{13}. Therefore, the percentage of acetate metabolized via succinic acid is

$$\frac{D \times C}{A \times B} \times 100 = \frac{0\ 28 \times 6 \times 100}{3\ 90 \times 0\ 62} = 70\ \% \tag{2}$$

By these approximate calculations it is estimated that about 70 per cent of the acetate disappearing is metabolized via the organic acids of the tricarboxylic acid cycle.

A similar calculation probably even less accurate has been made with the data from Experiment 1B to ascertain what fraction of the acetoacetate disappearing was metabolized via these same intermediates. In this experiment succinic acid was oxidized to fumaric and malic acids, the main products of metabolism. In a separate experiment it was shown that only a small proportion of the added metabolites was oxidized to carbon dioxide. The values from Experiment 1B to be substituted in equation (2) are as follows: (*A*) initial isotopic concentration in acetoacetate, 3.80 atom per cent excess; (*B*) millimoles of acetoacetate carbon metabolized, 4 $\times$ 0.34 = 1.36; (*C*) millimoles of succinic acid carbon added, 1.5 $\times$ 4 = 6.0; (*D*) isotopic concentration in the isolated fumarate, 0.30 atom per cent

excess. Therefore, the percentage of acetoacetate metabolized via fumaric acid is

$$\frac{0.30 \times 6 \times 100}{3.80 \times 1.36} = 35\ \%$$

According to these calculations at least 35 per cent of the acetoacetate disappearing is metabolized via the oxidative reactions of the tricarboxylic acid cycle. No attempt has been made to account for the remainder of acetoacetate metabolism, although undoubtedly the formation of β-hydroxybutyric acid, a reductive reaction, is an important consideration. In a similar experiment with pyruvate in liver mince, Wood *et al.* (34) have found that 45 per cent of the pyruvate disappearing could be accounted for by the formation of products of reductive reaction (*i.e.* lactate, malate, fumarate).

It is estimated that in Experiment 1B, 0.35 × 1.36 or 0.48 mM of acetoacetate carbon, and in Experiment 4, 0.70 × 0.62 or 0.43 mM of acetate carbon were metabolized via the oxidative reactions of the tricarboxylic acid cycle. Since the conditions in these two experiments were comparable, it is concluded that acetate and acetoacetate oxidations proceed by these reactions at approximately equal rates. These results are not in accord with the non-isotopic experiments of Weil-Malherbe and of Krebs and Eggleston, who could find no evidence for the oxidation of acetoacetate via the intermediates of the tricarboxylic acid cycle in sheep heart and kidney.

We wish to express our appreciation to Dr. Sidney Weinhouse and the Houdry Laboratories, Marcus Hook, Pennsylvania, for the isotopic analyses.

SUMMARY

It has been demonstrated that acetoacetate as well as acetate is oxidatively metabolized by way of the tricarboxylic acid cycle. After incubation of isotopic acetoacetate or acetate with kidney homogenates, it was found that isotopic carbon had been incorporated in α-ketoglutaric, succinic, or fumaric acid. There was no participation of CO_2 in this isotopic transfer. The amounts of acetate and acetoacetate metabolized by this process have been roughly estimated and the results indicate that this is an important metabolic pathway.

Chemical degradation of the isotopic α-ketoglutarate and fumarate indicated that the excess of C^{13} resides almost exclusively in the γ-carboxyl group of α-ketoglutaric acid. Citrate is therefore excluded as a significant intermediate.

The interrelationship of carbohydrate and fat oxidation is discussed and the conclusion drawn that metabolic intermediates of fat are con-

verted into CO_2 and H_2O by a process which is in part identical with the pathway of carbohydrate oxidation. A mechanism is proposed for ketone body oxidation which is consistent with the point of view that no "extra" carbohydrate is formed during the metabolism of fatty acids.

BIBLIOGRAPHY

1. Quastel, J. H., and Wheatley, A. H. M., *Biochem. J.*, **29**, 2773 (1935).
2. Edson, N. L., and Leloir, L. F., *Biochem. J.*, **29**, 2319 (1936).
3. Breusch, F. L., *Science*, **97**, 490 (1943); *Compt. rend. ann. arch. Soc. Turque sc. phys. et nat.*, **10**, 55 (1942–43).
4. Wieland, H., and Rosenthal, C., *Ann. Chem.*, **554**, 241 (1943).
5. Weil-Malherbe, H., *Nature*, **153**, 435 (1944).
6. Krebs, H. A., and Eggleston, L. V., *Nature*, **154**, 209 (1944).
7. Buchanan, J. M., Sakami, W., Gurin, S., and Wilson, D. W., *J. Biol. Chem.*, **157**, 747 (1945).
8. Hudson, B. E., Jr., Dick, R. H., and Hauser, C. R., *J. Am. Chem. Soc.*, **60**, 1960 (1938).
9. Donleavy, J. J., *J. Am. Chem. Soc.*, **58**, 1004 (1936).
10. Stotz, E., *J. Biol. Chem.*, **118**, 471 (1937).
11. Van Slyke, D. D., and Folch, J., *J. Biol. Chem.*, **136**, 509 (1940).
12. Blaise, E. E., and Gault, H., *Bull. Soc. chim.*, **9**, 451 (1911).
13. Veibel, S., *Bull. Soc. chim.*, **41**, 1410 (1927).
14. Perdrix, M. L., *Bull. Soc. chim.*, **17**, 100 (1897).
15. Allen, M. B., and Ruben, S., *J. Am. Chem. Soc.*, **64**, 948 (1942).
16. Friedemann, T. E., *J. Biol. Chem.*, **123**, 161 (1938).
17. Edson, N. L., *Biochem. J.*, **29**, 2082 (1935).
18. Evans, E. A., Jr., and Slotin, L., *J. Biol. Chem.*, **141**, 439 (1941).
19. Wood, H. G., Werkman, C. H., Hemingway, A., and Nier, A. O., *J. Biol. Chem.*, **139**, 483 (1941).
20. Lipmann, F., *Skand. Arch. Physiol.*, **76**, 255 (1937).
21. Evans, E. A., Jr., *Biochem. J.*, **34**, 829 (1940).
22. Krebs, H. A., in Nord, F. F., and Werkman, C. H., Advances in enzymology and related subjects, New York, **3**, 191 (1943).
23. Weinhouse, S., Medes, G., and Floyd, N. F., *J. Biol. Chem.*, **155**, 143 (1944).
24. Meyerhof, O., Ohlmeyer, P., Gentner, W., and Maier-Leibnitz, H., *Biochem. Z.*, **298**, 396 (1938).
25. Evans, E. A., Jr., A symposium on respiratory enzymes, Madison, 197 (1942).
26. Solomon, A. K., Vennesland, B., Klemperer, F. W., Buchanan, J. M., and Hastings, A. B., *J. Biol. Chem.*, **140**, 171 (1941).
27. Bloch, K., and Rittenberg, D., *J. Biol. Chem.*, **155**, 243 (1944).
28. Lipmann, F., and Tuttle, C. L., *J. Biol. Chem.*, **158**, 505 (1945).
29. Utter, M. F., Lipmann, F., and Werkman, C. H., *J. Biol. Chem.*, **158**, 521 (1945).
30. Buchanan, J. M., Hastings, A. B., and Nesbett, F. B., *J. Biol. Chem.*, **150**, 413 (1943).
31. Vennesland, B., Solomon, A. K., Buchanan, J. M., and Hastings, A. B., *J. Biol. Chem.*, **142**, 379 (1942).
32. Boxer, G. E., and Stetten, D., Jr., *J. Biol. Chem.*, **157**, 749 (1945).
33. Rittenberg, D., and Bloch, K., *J. Biol. Chem.*, **157**, 749 (1945).
34. Wood, H. G., Werkman, C. H., Hemingway, A., and Nier, A. O., *J. Biol. Chem.*, **142**, 31 (1942).

THE CHEMISTRY OF CYSTICERCUS FASCIOLARIS

III. THE COMPOSITION OF THE ACETONE-SOLUBLE FAT OF TRANSPLANTED RAT TUMORS INITIATED BY CYSTICERCUS FASCIOLARIS*

By SIDNEY F. VELICK

(*From the Department of Chemistry, Yale University, New Haven*)

(Received for publication, May 11, 1945)

The sarcomas arising in rat livers at the surface of the cysts surrounding the tapeworm larvae, *Cysticercus fasciolaris* (*Taenia taeniaformis*), have been extensively studied by Curtis, Dunning, and Bullock, (1). Recently in this laboratory, as part of a cooperative investigation sponsored by The International Cancer Research Foundation, a chemical investigation of the extractable components of the larvae was undertaken by Anderson and coworkers (2, 3). The opportunity has arisen in continuation of this project to examine the tumors caused by the parasite. As in the case of the larvae a large amount of carefully prepared tumor tissue was supplied by Dr. Dunning and Dr. Curtis.

Although the lipids of malignant mammalian neoplasia have been the subject of numerous investigations, they have not yet been subjected to a detailed chemical analysis. From the available evidence they appear in superficial character to resemble the lipids of normal tissues, although elevated levels of cholesterol esters (4) and of higher unsaturated acids determined as the polybromides (5) have been reported. Interest in tumor lipids resides in the possibilities for structural and metabolic peculiarities, and in the numerous reports of tumor-inducing properties in the lipid extracts of tissues from various sources. Samples of the fractions obtained in the present work have been reserved for biological study.

The fraction of the *Cysticercus* rat tumors designated acetone-soluble fat was completely soluble in cold ether and acetone and constituted 3.93 per cent of the dry weight of the tissue. Its composition and properties are summarized in Table I. Expressed as per cent of the dry weight of the tumor tissue, the amounts of fat and unsaponifiable material fall within the rather wide limits observed in normal organ tissues. In accordance with previous reports the iodine number of the fatty acids was high but the cholesterol distribution was not unusual. About 30 per cent of the fatty acids was free. The remaining acids were combined as simple glycerides. There was also present a small amount of phosphatide which was not removed during the purification.

* This work was aided by a grant from The International Cancer Research Foundation.

The fatty acids were fractionated by way of the lead salts in alcohol and ether and the lithium salts in acetone, and were then converted to the methyl esters and fractionally distilled. In Table II is presented the

TABLE I

Composition of the Acetone-Soluble Fat of Transplanted Rat Tumors Initiated by Cysticercus fasciolaris

Substance	Per cent of acetone-soluble fat
Total fatty acids	66.3
Free " " calculated as oleic acid	20.3
Total cholesterol	24.4*
Free "	18.4*
Unidentified unsaponifiable material	4.5
Glycerol	4.4
Glycerophosphoric acid	0.21
Choline	0.28
Acid No. of fat	40.1
Volatile fatty acids	Trace
Iodine No. of fat	91.9
" " " total acids	96

* Expressed as per cent of the total dry weight of the tumor tissue, these values are as follows: total cholesterol 0.95 and free cholesterol 0.72 per cent, respectively.

TABLE II

The Fatty Acids of the Acetone-Soluble Fat of Transplanted Rat Tumors Initiated by Cysticercus fasciolaris

Acid	Per cent of total acids
Myristic and lower acids	1
Palmitic acid	23
Stearic "	14
Eicosanoic and higher acids	1
Lower unsaturated acids	Trace
Hexadecenoic acid	3.5
Oleic acid	28
Linoleic acid	10.5
Eicosenoic acids	13
Docosenoic acids	6

observed distribution of the fatty acids. The major saturated acids were isolated in sufficiently pure form for positive identification. The unsaturated acids were separated into groups homogeneous with respect to

the number of carbon atoms but not with respect to the number of double bonds. Although the amounts of unsaturated C_{20} and C_{22} acids greatly exceed the values for normal rat carcass glycerides (6), they correspond in certain respects to those reported for ox, pig, and sheep liver glycerides (7) and to the total carcass acids of rats fed high fat diets, particularly diets containing 40 per cent cod liver oil (8). It should be noted that, although *Cysticercus* rat tumors arise in the liver, they do not represent malignant transformations of functional liver cells, and hence comparison with liver glycerides must be interpreted with caution.

As far as could be determined the unsaturated acids of the C_{18} series consisted entirely of oleic and linoleic acids. Linoleic acid was isolated and identified through its crystalline tetrabromide. Oleic acid was not isolated in pure form but its presence in the octadecenoic acid fraction was demonstrated. The unsaturated C_{20} acids were a mixture of components containing one to four double bonds and the C_{22} group contained acids with four or more double bonds.

EXPERIMENTAL

The tumor tissue consisted entirely of transplants of tumors initiated in rats by the administration of the eggs of *Taenia taeniaformis*. Rats four to seven months of age of the same inbred strains as the primary hosts were employed. They were maintained on a stock diet of bread dipped in milk, yellow corn, and a variety of greens. When the tumors reached an approximate weight of 50 gm., from an implant of 2 mg. in the subcutaneous tissue, they were excised, dissected free of normal and necrotic tissue, cut into small pieces, and stored in alcohol. Histologically they were of three types, in which the cells were predominantly spindle cells, polymorphous cells, and plasma cells respectively.

Before extraction the coarsely minced tissue was reduced to a fine pulp under alcohol in a Waring blendor. Extraction was carried out by allowing the pulp to stand with occasional shaking under a series of solvents at room temperature. The pulp was extracted in this manner twice with 5 liters of alcohol, once with 5 liters of acetone, and twice with 4 liters of ether. The insoluble residue was then extracted three times with 4 liters of a boiling 3:1 methanol-chloroform mixture. After each extraction the suspension was filtered by suction through washed canvas and filter paper. The clear solutions were concentrated under reduced pressure at a bath temperature of 45–50°. The alcohol extracts, which contained most of the tissue water, were concentrated until most of the alcohol was removed and were then extracted with ether. Subsequent extracts, after filtration and removal of the solvent, were dissolved in 2 to 3 volumes of ether. In each case the ether solution was chilled overnight and cen-

trifuged to remove ether-insoluble material, and the clear solutions poured into 3 to 4 times their volume of cold acetone. The resulting precipitates were filtered off, washed with acetone, and set aside for separate study. The filtrates and washings were combined, evaporated *in vacuo*, and dissolved in ether. A small flocculent precipitate appeared on chilling and was filtered off. The filtrate was shaken twice with water to remove water-insoluble impurities and the ether layer was dried over sodium sulfate. Only a trace of precipitate appeared when the dried ether solution was poured into acetone. The acetone was distilled from the filtered solution under reduced pressure and the residual reddish oil was dried *in vacuo* over phosphorus pentoxide to constant weight.

The aqueous washings and the water-soluble portions of the alcohol extracts were taken to dryness *in vacuo* with the addition of amyl alcohol to prevent excessive foaming. The residue was extracted repeatedly

TABLE III

Crude Fractions of the Transplanted Rat Tumors Initiated by Cysticercus fasciolaris

Fraction	Weight	Per cent of total dry tumor
	gm.	
Acetone-soluble fat	63	3.93
Acetone-insoluble lipids	40	2.49
Ether-insoluble lipids	6	0.37
Water-soluble fraction of crude lipid extracts		
Alcohol-soluble	32	2.0
Alcohol-insoluble	116	7.2
Residue	1346	83.9
Total dry weight	1603	

with hot absolute alcohol and the insoluble portion rubbed under alcohol until it was reduced to a powder. The alcohol-soluble portion on removal of the solvent formed a dark gum. Table III gives a summary of the crude extracts.

Saponification of the Fat—The fat was saponified with an excess of potassium hydroxide in 90 per cent alcohol on the steam bath for 1 hour. Most of the alcohol was removed *in vacuo* and the residue diluted with water and extracted with ether. The ether solution was washed with water, evaporated, and the residue resaponified for 4 hours. The small amounts of additional soaps so obtained were added to the main batch and the fatty acids were isolated in the usual way. The water-soluble fraction was neutralized and evaporated to dryness *in vacuo*. The unsaponifiable fraction was likewise freed of solvent and reserved for subsequent examination.

Preliminary Fractionation of the Acids—The separation of the saturated and unsaturated acids was undertaken before distillation of the esters. Although this procedure reduced the volume of material available for a single distillation and hence decreased the distillation accuracy, it eliminated the need for separate salt fractionations on the numerous small distillation fractions. It also reduced to a minimum the amount of time in which the more labile unsaturated esters were subjected to distillation conditions.

The saturated acids were isolated from the crude mixture by two precipitations of the lead salts from alcohol according to the Twitchell procedure (9) and the lead salts were extracted with warm ether. The resulting insoluble salts were filtered off, washed with ether, and converted to the free acids. Diazomethane in ether solution (10) was used as the esterifying agent. The excess reagent and solvent were distilled off after standing for 1 hour at room temperature.

The alcoholic filtrate from the lead salt precipitation of the saturated acids was combined with the alcohol and ether washings and evaporated *in vacuo*. From the residue the free unsaturated acids were obtained by shaking with dilute hydrochloric acid and ether. The resulting acids were dissolved in 4 volumes of acetone and neutralized to phenolphthalein with 10 per cent lithium hydroxide in water. Acetone was added until a concentration of 95 per cent was reached and the mixture was chilled overnight. The precipitate was filtered, and washed with cold acetone. This procedure separated the major portion of the oleic and linoleic acids as the insoluble lithium salts and left the higher unsaturated acids in solution (11). The free acids were recovered from both of the lithium salt fractions and esterified with diazomethane.

The results of the salt fractionations are summarized in Table IV. It can be seen that the lithium salt fractionation effectively isolated the higher unsaturated fraction. As shown by the subsequent distillation, the molecular weights of the acids forming soluble lithium salts were higher than those in the other fractions. However, the average value observed by titration of a sample of the undistilled material was misleadingly high because impurities tended to be concentrated in that fraction.

Distillation of the Methyl Esters—The methyl esters of the three groups of acids resulting from the salt fractionation were fractionally distilled at a pressure of 1 mm. The column employed was originally constructed in a slightly different form for the distillation of small amounts of esters of branched chain fatty acids and has been briefly described (12). It is shown diagrammatically in Fig. 1, together with the essential details of construction.

In carrying out a distillation the system was evacuated to the desired

pressure and the pot, *B*, heated in a Woods metal bath until the lower part of the spiral packing was wet. The jacket temperature was then slowly raised until the reflux reached the top of the column. The temperature of the supplementary heater was slowly raised until distillation occurred at a rate of 0.2 to 0.5 cc. per hour. Both jacket and pot temperatures were adjusted so that distillation slowed down and stopped when they were lowered a few degrees. In the transition region between components the jacket and pot temperatures were raised by small increments and small fractions taken until a steady state was again established.

The progress of the distillation was followed by measurements of refractive index. Saturated esters isolated in pure form and unsaturated esters essentially homogeneous with respect to the number of carbon atoms represent plateaus in the distillation curve in which the refractive index was determined at several points and remained constant or showed only slight drift. The saturated members were identified by melting

TABLE IV

The Properties of the Fractions Obtained by Salt Fractionation of the Total Acids from 17.42 Gm. of Acetone-Soluble Fat

Fraction No.	Source	Weight	Per cent of total acids	Iodine No.	Neutral equivalent
		gm.			
1	Ether-insoluble lead salts	4.39	38.2	1.9	265
2	Acetone-insoluble lithium salts	4.30	36.1	124	283
3	Acetone-soluble lithium salts	2.70	25.7	242	361

point and refractive index of the methyl ester and by the neutral equivalent and melting point of the free acid. Iodine numbers of the unsaturated esters were determined by the method of Yasuda (15). Samples were then hydrogenated with platinum oxide catalyst, and the reduced esters and corresponding acids characterized in the manner described above. Saponification of the esters was effected with 10 per cent potassium hydroxide in 90 per cent alcohol on the steam bath. The free acids were crystallized once from a small volume of acetone. Titrations were carried out in benzene with alcoholic potassium hydroxide to a phenolphthalein end-point.

As far as can be judged from an evaluation of the procedures the *relative* accuracy of the distillation figures is ±5 per cent or better for the major components. In making the calculations the small per cent of homologous impurity in the constant boiling fractions has been neglected. There were small polymerized distillation residues which were also neglected, since their source could not be accurately assigned. Presumably they

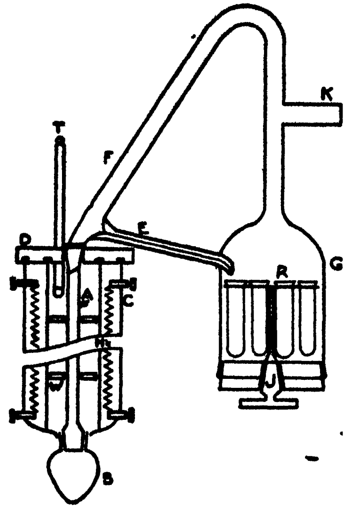


FIG. 1. Diagram of apparatus used in the fractional distillation of small amounts of methyl esters. The column, A, is of 4 mm. internal diameter and 30 cm. long. It is packed with a close fitting spiral, 6 to 8 turns per inch, of No. 22 stainless steel wire or glass of corresponding gage, as introduced by Podbelniak (13). The column is attached to the still head, F, by a 12/30 ground glass joint and to the pot, B, of 20 cc. capacity, by a 14/20 ground glass joint. These joints are lubricated with bicycle graphite. The jacket, C, consists of concentric tubes of 25 and 50 mm. diameter respectively. The inner jacket tube rests on a constriction at the bottom of the outer tube, and is centered at the top by a grooved Transite disk, D. Through the disk, D, is inserted a thermometer, T, and the junction of the column and still head. In the outer annular space of the jacket are strung 3 coiled springs, H, of No. 24 nichrome wire attached to binding posts inserted through the outer jacket tube. These strands, which contain a total of 12 feet of wire, are connected to form a single heating element controlled by a variable transformer. The inner annular space, between the column, A, and the inner jacket tube, contains two asbestos baffles, W, which help maintain the temperature gradient. The still head is similar to one employed on a column of slightly larger capacity by Klenk and Schuwirth (14). It is attached to the column by a 12/30 male joint, which is cut at a 45° angle at the bottom to prevent a tendency to flood at that point. Distillate is taken off through the capillary, E, which has a 0.5 mm. bore. The vacuum line is attached at K. In order to facilitate control of distillation rate and prevent solidification in the capillary the segment of the head between the disk, D, and the capillary is insulated with asbestos. The asbestos is wound with a few turns of nichrome wire, which is continued around the capillary as far as the receiver where it is secured by a binding strip. This supplementary heater, which is not shown in the diagram, is controlled by a variable transformer. The receiver, G, is 60 mm. in diameter and extends 90 cm. below the junction of the capillary. It is fitted with a No. 12 rubber stopper through the center of which is a 14/30 ground glass joint, J. The eight receiving tubes, R, are of 1 cc. capacity and set in holes in the perimeter of a copper disk mounted on a shaft through J. The rubber stopper should be lubricated with glycerol before each run.

arose chiefly from the higher unsaturated esters, and hence the figures for these components are probably too low. The compositions of the three main groups of acids were determined separately and multiplied by the appropriate factors to give the figures in Table II.

The Saturated Acids—The results for this group are shown in Table V. As in most mammalian fats palmitic acid was the predominant saturated acid. Together with stearic acid it constituted more than 95 per cent of the saturated fraction. The esters of these acids were obtained in comparatively pure form, as indicated by the physical constants. Lower and higher members of the saturated series did not occur in amounts sufficient for purification and positive identification. From the boiling range in which they were obtained it is assumed that they were chiefly myristic and *n*-eicosanoic acids.

TABLE V

The Saturated Acids and Their Methyl Esters

Fraction No.	Acid composition	Methyl esters			Free acids	
		Weight	Refractive index, n_D^{45}	m.p.	m.p.	Neutral equivalent
		gm.		°C.	°C.	
1	Palmitic and lower acids	0.81	1.4301–1.4315			245
2	" acid	4.49	1.4318	28.8	62.1	257*
3	" and stearic acids	1.82	1.4321–1.4344		55	272
4	Stearic acid	2.89	1.4348	38.6	69.2	284†
5	" and higher acids	0.33	1.4355–1.4379		67	295

* Palmitic acid, m.p. 62.9°, mol. wt. 256; methyl palmitate, m.p. 29.8°.
† Stearic acid, m.p. 69.8°, mol.wt. 284; methyl stearate, m.p. 38.7°.

Unsaturated Acids Which Formed Lithium Salts Insoluble in Acetone—The crude esters of this fraction weighed 9.7 gm. and represented acids containing 14 to 22 carbon atoms, Table VI. Esters of the acids containing 14 or fewer carbon atoms occurred in amounts too small for purification. The first constant boiling fraction consisted of esters of C_{16} acids. A sample weighing 304 mg. was hydrogenated in methanol solution. The saturated ester obtained after filtration of the platinum oxide and removal of the solvent melted at 27.8°. On saponification it yielded 268 mg. of palmitic acid, m.p. 61.8°. From the yield of palmitic acid obtained and the iodine number of the original unsaturated ester it may be concluded that the C_{16} acid was palmitoleic acid containing about 16 per cent of palmitic acid.

The major fraction in this group consisted of esters of the unsaturated C_{18} acids. The iodine number and index of refraction of the esters indicated

a mixture of 25 to 30 per cent of methyl linolate and 70 to 75 per cent of methyl oleate. A portion of the ester was saponified and the free acids isolated and freed of traces of solvent and color by molecular distillation at a pressure of 0.05 mm. A sample of the distilled acids weighing 2.95 gm. was brominated in 25 cc. of low boiling petroleum ether at 0°. The crystalline precipitate weighed 0.82 gm. and melted at 112.5°. After one crystallization from ligroin the melting point rose to 113.5° and mixed with an authentic sample of tetrabromostearic acid (m.p. 114°); prepared from corn oil, it melted at 114°. If the yield of tetrabromide was 50 per cent, then linoleic acid constituted 25.9 per cent of the fraction under in-

TABLE VI

Unsaturated Acids with Lithium Salts Insoluble in Acetone

Fraction No.	No. of carbon atoms in acids	Methyl esters			Reduced acids	
		Weight	Refractive index, n_D^{20}	Iodine No	m.p	Neutral equivalent
		gm			°C.	
1	Up to 16	0.14	1.4441	55	43	236
2	16	0.55	1.4499	70	61.8	258*
3	16–18	0.89	1.4499–1.4561	93	57	272
4	18	7.05	1.4550–1.4558	108	69.4	284†
5	18–20	0.45	1.4558–1.4620	128	64	292
6	20–22	0 52	1.4620–1.4730	144	71	320

* Palmitic acid, m.p. 62.9°, mol. wt. 256.

† Stearic acid, m.p. 69.8°, mol. wt. 284.

vestigation, which falls within the range indicated by the iodine number and index of refraction of the methyl esters.

Analysis—14.61 mg. bromo acid: 18.10 mg. AgBr

$C_{18}H_{32}O_2Br_4$. Calculated, Br 53.3; found, 52.7

There was no ether-insoluble polybromide, and hence no detectable linolenic acid. The remainder of the C_{18} acid must therefore have been oleic acid together with possible traces of stearic acid. An attempt was made to characterize the oleic acid by ozonization. 2.0 gm. of the unsaturated C_{18} acids were dissolved in 10 cc. of chloroform and an excess of ozone passed through the solution at 0°. The solvent was taken off at 35° *in vacuo* and the ozonides decomposed by stirring for 1 hour at 80° in water containing a few per cent of hydrogen peroxide. The product was extracted from the aqueous suspension with ether, washed with small volumes of dilute ferrous ammonium sulfate and water, dried over sodium sulfate, and evaporated. The residue was extracted with cold

low boiling petroleum ether and the insoluble portion, which weighed 0.4 gm., was recrystallized from water after filtering off a small amount of water-insoluble gum. There was obtained 0.25 gm. of azelaic acid which melted at 101°. On recrystallization from water it melted at 103° and had a neutral equivalent of 95; pure azelaic acid, with a neutral equivalent of 94, melts at 106°. The yield of purified azelaic acid obtained, although poor, was comparable with that obtained in control experiments on known acids and indicates that the monounsaturated acid was ordinary oleic acid.

The two final distillation fractions in this group were mixtures as indicated by the neutral equivalents of the free acids obtained after reduction and saponification. Both fractions gave small amounts of ether-insoluble

TABLE VII

The Unsaturated Acids with Lithium Salts Soluble in Acetone

Fraction No.	No. of carbon atoms in acids	Methyl esters			Reduced esters	Reduced acids	
		Weight	Refractive index, n_D^{20}	Iodine No.	m.p.	m.p.	Neutral equivalent
		gm.			*°C.*	*°C.*	
1	18	0.85	1.4610	132	38.1	69.1	284*
2	18–20	1.27	1.4610–1.4785				296
3	20	1.04	1.4785–1.4789	257	44.5	74.6	314†
4	20–22	0.78	1.4789–1.4876				322
5	22	0.60	1.4877–1.4889	288	51	77	336‡

* Methyl stearate, m.p. 38.7°; stearic acid, m.p. 69.8°, mol. wt. 284.

† Methyl eicosanoate, m.p. 45.8°; eicosanoic acid, m.p. 75.6°, mol. wt. 312.

‡ Methyl docosanoate: m.p. 52.7°; docosanoic acid, m.p. 79.9°, mol. wt. 340.

bromides. The final fraction, which was approximately 57 per cent C_{20} and 43 per cent C_{22}, had an iodine number of 144. This was less than the calculated value for two double bonds ($C_{20}H_{35}O_2CH_3$, iodine number 158). Since there were no detectable saturated acids present, the unsaturated acids contained one or two double bonds or both.

Acids Which Formed Lithium Salts Soluble in Acetone—Esters of unsaturated acids of the C_{18}, C_{20}, and C_{22} series were present in this group. They are listed in Table VII together with the intermediate fractions. No trace of an acid lower than C_{18} could be detected. The only detectable reduction product of the octadecenoic esters was methyl stearate. From 110 mg. of the free acid there were obtained, on bromination in petroleum ether at 0°, 120 mg. of polybromide which melted at 113° after one recrystallization from ligroin. These results indicate that the fraction was largely

linoleic acid. The low iodine number cannot be explained with the available data. Corresponding acid fractions from animal sources have frequently been observed to give low rather than high yields of the crystalline tetrabromide, a result which has been attributed to the presence of isomeric acids (16, 17).

Analysis—19.03 and 22.78 mg. of tetrabromide: 22.55 and 26.86 mg. AgBr
$C_{18}H_{32}O_2Br_4$. Calculated, Br 53.3; found, Br 50.4, 50.2

The second constant boiling fraction, as shown by its hydrogenation product, consisted of esters of unsaturated acids of the C_{20} series. Calculated from the iodine number the average number of double bonds in this group was 3.2 per molecule. It therefore consisted of esters containing four or more double bonds, together with esters of lower unsaturation, the presence of which had previously been demonstrated in the acids which formed acetone-insoluble lithium salts. The polybromide addition product, a mixture, was insoluble in ether and decomposed on heating. It contained slightly more than 6 bromine atoms per molecule.

Analysis—90 mg. C_{20} acid yielded 320 mg. of polybromide; 19.47 and 20.80 mg. polybromide: 29.09 and 30.98 mg. AgBr
$C_{20}H_{34}O_2Br_6$. Calculated, Br 61.07; found, 63.58, 63.38

The final distillation fractions still showed a drift in index of refraction. From the final 0.6 gm. of esters there was obtained after hydrogenation and saponification an acid which corresponded in properties with *n*-docosanoic acid containing about 8 per cent of *n*-eicosanoic acid, as indicated by the neutral equivalent and low melting point. The unsaturated ester contained approximately four double bonds per molecule.

The Unsaponifiable Fraction—A sample of the acetone-soluble fat weighing 0.1500 gm. was dissolved in 5 cc. of 95 per cent alcohol and treated with 12 cc. of a 1 per cent solution of purified digitonin. The precipitate was filtered through a tared sintered glass funnel after standing for 12 hours, washed with a few cc. of 80 per cent alcohol, and dried at 110°. The precipitate weighed 0.110 gm., which corresponded to 0.0275 gm. of free cholesterol or 18.4 per cent of the acetone-soluble fat.

A sample of the fat which weighed 17.42 gm. yielded on saponification in the manner previously described 5.02 gm. of unsaponifiable material which corresponded to 28.8 per cent of the acetone-soluble fat. From this material there were obtained by fractional crystallization from alcohol 4.04 gm. of crude cholesterol. After treatment with norit and one recrystallization from alcohol it melted at 146° $[\alpha]_D^{23} = -40.72°$ (0.0980 gm. in 3.0 cc. of chloroform). The acetate was prepared by refluxing a small sample for 40 minutes in 5 times its weight of acetic anhydride. After

recrystallization from alcohol and acetone the acetate melted at 116.2° and gave no depression in mixed melting point with cholesterol acetate.

The residual unsaponifiable material obtained from the final cholesterol mother liquors was a reddish brown solid with an iodine number of 76.5.

The Water-Soluble Fraction—The dried water-soluble fraction of the fat, prepared as described in a preceding section, gave a negative Molisch test. In order to remove the potassium chloride the material was extracted with hot absolute alcohol and filtered. The alcoholic solution was evaporated and reextracted in the same manner and the process was repeated again. The final alcoholic solution was evaporated and the residue was dissolved in water and passed through small columns of ion exchange resins, Amberlite 1-R-100 and 1-R-4 prepared as described by McCready and Hassid (18). From the final filtrate there was obtained a clear almost colorless sample of glycerol which yielded a tribenzoate melting at 75.8°, mixed melting point 76°. The glycerol so obtained from 31 gm. of fat weighed 1.36 gm. When corrections are made for the free fatty acids and the unsaponifiable material, the recovery of glycerol is 86 per cent of the theoretical.

The 1-R-4 resin was eluted with 5 per cent ammonia and the eluate evaporated to dryness *in vacuo* and dissolved in 5 cc. of water. A small excess of barium hydroxide solution was added and the resulting few mg. of inorganic precipitate filtered off. To the filtrate were added 2 volumes of alcohol. The precipitate was centrifuged off and reprecipitated twice from small volumes of water by addition of alcohol. There were obtained 116 mg. of a white powder which was shown by analysis to be barium glycerophosphate. For the determination of glycerol the apparatus of Elek (19) was employed.

Analysis—$C_3H_7O_6PBa$

Calculated.	P 10.09,	Ba 44.7,	glycerol 30.0
Found.	" 9.23,	" 42.6,	" 28.1

The 1-R-100 resin was eluted with 4 per cent hydrochloric acid and the eluate evaporated to dryness *in vacuo*. The residue was dissolved in absolute alcohol and treated with a small excess of an alcoholic solution of chloroplatinic acid. There were obtained 221 mg. of the orange-colored choline chloroplatinate which gave the characteristic odor of trimethylamine on ignition. For analysis the salt was recrystallized from dilute alcohol.

Analysis—$(C_5H_{14}NOCl)_2PtCl_4$. Calculated, Pt 31.7; found, 30.8

SUMMARY

1. The extraction and partial fractionation of the lipids of transplanted rat tumors initiated by *Cysticercus fasciolaris* have been described.

2. The acetone-soluble fat has been examined. It contains ordinary glycerides, a large amount of free fatty acids, and 28.8 per cent of unsaponifiable material, chiefly cholesterol.

3. The fatty acid distribution has been determined by salt fractionation and fractional distillation of the methyl esters. It is characterized by a high content of linoleic acid and unsaturated acids of the C_{20} and C_{22} series.

BIBLIOGRAPHY

1. Curtis, M. R., Dunning, W. F., and Bullock, F. D., *Am. J. Cancer*, **17**, 894 (1933); **21**, 86, 554 (1934).
2. Salisbury, L. F., and Anderson, R. J., *J. Biol. Chem.*, **129**, 505 (1939).
3. Lesuk, A., and Anderson, R. J., *J. Biol. Chem.*, **139**, 457 (1941).
4. Bierich, B., and Lang, A., *Z. physiol. Chem.*, **216**, 217 (1933).
5. Smedley-MacLean, I., and Nunn, L. C. A., *Biochem. J.*, **35**, 983 (1941).
6. Longenecker, H. E., *Chem. Rev.*, **29**, 201 (1941).
7. Hilditch, T. P., and Shorland, R. B., *Biochem. J.*, **31**, 1499 (1937).
8. Channon, H. J., Jenkins, G. N., and Smith, J. A. B., *Biochem. J.*, **31**, 41 (1937).
9. Twitchell, E., *J. Ind. and Eng. Chem.*, **13**, 806 (1921).
10. Organic syntheses, New York, coll. **2**, 165 (1943).
11. Tsujimoto, M., *J. Chem. Ind.*, Japan, **23**, 1007 (1920).
12. Velick, S. F., *J. Biol. Chem.*, **152**, 533 (1944).
13. Podbelniak, W., *Ind. and Eng. Chem., Anal. Ed.*, **3**, 177 (1931).
14. Klenk, E., and Schuwirth, K., *Z. physiol. Chem.*, **267**, 260 (1941).
15. Yasuda, M., *J. Biol. Chem.*, **94**, 401 (1931-32).
16. Hilditch, T. P., and Stainsby, W. J., *Biochem. J.*, **29**, 90 (1935).
17. Cramer, D. L., and Brown, J. B., *J. Biol. Chem.*, **151**, 427 (1943).
18. McCready, R. M., and Hassid, W. Z., *J. Am. Chem. Soc.*, **66**, 560 (1944).
19. Elek, A., *Ind. and Eng. Chem., Anal. Ed.*, **11**, 174 (1939).

ANTIBIOTIC SUBSTANCES PRODUCED BY PSEUDOMONAS AERUGINOSA*

By EDWIN E. HAYS, IBERT C. WELLS, PHILIP A. KATZMAN, C. K. CAIN, FRANCIS A. JACOBS, SIDNEY A. THAYER, AND EDWARD A. DOISY

(From the Laboratory of Biological Chemistry, St. Louis University School of Medicine, St. Louis)

W. L. GABY, E. C. ROBERTS, R. D. MUIR, C. J. CARROLL, AND L. R. JONES

(From the Department of Bacteriology, St. Louis University School of Medicine, St. Louis)

AND NELSON J. WADE

(From the Department of Biology, St. Louis University, St. Louis)

(Received for publication, May 10, 1945)

In 1877 Pasteur (1) reported that animals injected with an inoculum containing a mixture of *Bacillus anthracis* and certain other common bacilli failed to develop anthrax. 10 years later, in 1887, Emmerich (2) during a demonstration before his class accidentally discovered that a guinea pig which had been previously injected with a culture of *Streptococcus erysipelatis* did not develop cholera when inoculated with *Vibrio cholerae*. He immediately recognized the significance of this discovery and proceeded to prevent anthrax in experimental animals by administering cultures of *Streptococcus erysipelatis* prior to the injection of a *B. anthracis* culture. 2 years later Bouchard (3) observed that the injection of small quantities of cultures of *Pseudomonas aeruginosa* (*Bacillus pyocyaneus*) prevented the development of anthrax in rabbits which had been previously injected with a virulent culture of *B. anthracis*. Woodhead and Wood in 1889 (4) carried the work a step farther by showing that sterilized cultures of *Ps. aeruginosa* had the same effect. During the same period *in vitro* studies by Charrin and Guignard (5) and Blagovestchensky (6) demonstrated that *Ps. aeruginosa*, or the filtrates from cultures of this organism, would destroy *B. anthracis*. Furthermore, von Freudenreich (7) showed that filtrates from 4 to 6 week-old cultures of *Ps. aeruginosa* would not support the growth of several pathogenic microorganisms. Emmerich and Löw (8) used a cell-free culture fluid of *Ps. aeruginosa*, which had been concentrated to one-tenth of its original volume, to prevent the development of anthrax in rab-

* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and St. Louis University.

bits which had been inoculated with *B. anthracis*. *In vitro* this product destroyed *Corynebacterium diphtheriae*, staphylococci, streptococci, pneumococci, gonococci, *Vibrio cholerae*, and *Shigella paradysenteriae*. Owing to certain enzymatic activities of this product Emmerich named it pyocyanase. Although these early observations led to the commercial production of the product, pyocyanase, which was extensively used in the therapy of diphtheria, grippe, and meningitis, its use gradually fell into disrepute, probably because the concerns engaged in its manufacture failed to supply a potent product.

During the course of the next few years there was considerable controversy over the nature of pyocyanase. Although Emmerich had shown its heat stability, he was reluctant to concede that the antibiotic activity was not enzymatic in nature (Klimoff (9) and Dietrich (10)). Furthermore, he was unwilling to admit that the bactericidal effect was due to the lipids derived from the organism. Later several investigators, Raubitschek and Russ (11), Fukuhara (12), and Ohkubo (13), not only showed that nearly all of the bactericidal material could be extracted from the commercial pyocyanase with lipid solvents but that extraction of the cells of *Pseudomonas aeruginosa* with alcohol yielded a potent preparation, a finding which conforms with our observation that the antibiotic substances obtained from the cells are readily soluble in organic solvents. Subsequent investigators have worked chiefly with lipid extracts.

In 1911, Fukuhara (12) showed that exposure of the viruses of vaccinia, rabies, and hen pest to the pyocyanase lipid prevented the appearance of the respective diseases in rabbits or chickens inoculated with the treated virus.

In spite of the promising results obtained by the early workers, the clinical use of pyocyanase was more or less abandoned. Bocchia (14) found that an aqueous solution prepared by passing the culture fluid through a Berkefeld filter, a process similar to that introduced by Emmerich and presumably used in commercial production, had only a weak therapeutic action, and, therefore, could not be recommended for clinical practice. This experience was confirmed by Wagner (15). Such a filtrate would contain little antibiotic potency, as most of the active material was removed with the cell bodies. Furthermore, in 1935, Kramer (16) showed that some strains of *Pseudomonas aeruginosa* do not produce pyocyanase and that actively producing strains may lose this ability.

Following the work of Raubitschek and Russ, Ohkubo, and Fukuhara, several investigators studied the lipids of *Pseudomonas aeruginosa*. Hosoya (17), in 1928, obtained a crystalline product which was soluble in organic solvents. Although some qualitative reactions were described, the only evidence of isolation was the crystalline character. In the form of a sus-

pension this product killed *Corynebacterium diphtheriae*, *Shigella paradysenteriae*, *Bacillus anthracis*, *Staphylococcus albus*, and certain streptococci. Gundel and Wagner (18) concluded that the antibiotic potency of lipid extracts of the organism was due to their fatty acid content. Hettche (19–23) reported that commercial pyocyanase possessed little activity. The lipid which he obtained from dried *Pseudomonas aeruginosa* was fractionated and the separate fractions tested. He concluded that the bactericidal property was due chiefly to liquid fatty acids. Birch-Hirschfeld (24) also studied the production and properties of pyocyanase and obtained a crystalline product which was not identified. The hemolytic activity, bactericidal activity, and surface tension of a solution of this product were comparable. Schoental (25) isolated pyocyanine (previously studied by several investigators, see Hettche (19)), α-oxyphenazine (see Wrede and Strack (26–28)), and a pale yellow oil which possessed antibiotic properties. Although this product was studied both bacteriologically and chemically, it was not fractionated to obtain pure crystalline antibiotics.

At the time Schoental's paper directed our attention to pyocyanase it had been reported (*a*) that *Pseudomonas aeruginosa* produces a substance that destroys several kinds of bacteria and viruses; (*b*) that the antibiotic substances belong to the lipid class or at least they are soluble in lipid solvents; (*c*) that strains of *Pseudomonas aeruginosa* may not produce these antibiotics and that a strain may lose its capacity to produce them; (*d*) that pyocyanase had been used successfully in the treatment of certain bacterial diseases. In spite of all the information which had been accumulating during the last half century, the antibiotic substances had never been isolated and characterized chemically and bacteriologically.

Pseudomonas aeruginosa Used in Our Work

Two cultures of *Pseudomonas aeruginosa*, obtained from lesions infected with this organism, have been employed for the production of the Pyo[1] compounds in our investigation. One of these, P-SLU, a stock culture maintained in our laboratory for the past several years, produces acid without gas from glucose, but fails to attack xylose and galactose. It liquefies gelatin slowly and produces a chloroform-soluble, blue-green pigment.

The use of the culture, P-SLU, was discontinued when the crude extracts

[1] Since our work clearly shows that the antibiotics of *Pseudomonas aeruginosa* are not enzymes, we are unwilling to use the old name, pyocyanase, with its connotation of enzymatic character. Due to our inability at present to give names based on chemical constitution, we shall call the compounds in this report Pyo I, Pyo II, Pyo III, and Pyo IV, for historical reasons and for the purpose of correlating the older and later work.

failed to produce the usual yield of active material, although a study of the morphological and cultural characteristics of this organism indicated that the culture had not undergone any other recognizable alteration in these respects.

The culture more recently employed, P-CC, was isolated from a patient with chronic cystitis. These organisms display one polar flagellum and produce acid without gas from glucose, galactose, and xylose. They are regarded as being actively proteolytic, since they liquefy gelatin in less than 24 hours and completely digest litmus milk in less than 48 hours. A water-soluble, green fluorescent pigment is produced. Crude extracts of the cells of 5 week-old cultures show high antibacterial titers. Throughout this study, the stock culture has been preserved by evaporation from the frozen state and subcultures are obtained periodically from this source.

A comparative study of the two cultures was made to determine the cause of the sudden decline in the extractable potency of culture P-SLU. These two cultures were apparently identical in all respects with the exception of pyocyanine production and the slight variation in biochemical reactions. It was not until a comparative study of the respective colonies was made that a striking difference was observed. Culture P-CC presented such a confusing variety of colony forms that it appeared on first inspection to be contaminated. Pigmentation varied from green fluorescent to white. Culture P-SLU was composed of a blue-green, pigmented colony and a larger, smooth, non-pigmented variety.

Lartigau in 1898 (29), Hadley (30), and Kramer (16) called attention to the fact that *Pseudomonas aeruginosa* is subject to variation under conditions of laboratory cultivation. Many strains lose their ability to produce the chloroform-soluble, blue-green pigment. When such strains are plated out, it is found that all of the colonies do not produce the blue-green color, but some merely possess a yellowish fluorescent pigment. It appears fairly certain to us that this variation in pigment production alone accounts for much of the confusion encountered in the literature concerning the identification and classification of this bacillus. It is also possible that, with the change in ability to produce pigment, there is a corresponding change in other characteristics as well. Therefore, a detailed examination of the various colony forms was undertaken in an attempt to determine these changes, if any, for the purpose of isolating a strain capable of producing a greater and consistent yield of the Pyo compounds.

By selective colony transfers and dissociation studies (to be described in detail elsewhere) it was possible to separate the P-CC culture into strains. In this manner we have succeeded in isolating a rough non-pigmented variety responsible for Pyo production, and a strain responsible for pyocyanine production which does not produce Pyo. When a colony contains a mix-

ture of these two varieties, the appearance of the colony is primarily that of the pyocyanine-producing one, but when this mixture is separated there is a striking dissimilarity in colony morphology of the two constituents. By serological methods we have found that the colony morphology may be correlated also with the antigenic pattern.

Cellular extracts of 5 week-old cultures of the pyocyanine-producing strain yield only negligible amounts of the Pyo compounds. From all of these results, it becomes possible to explain the decline in the ability of culture P-SLU to produce the Pyo compounds as being due to the loss of the potentiality for development of the round non-pigmented colony type.

From our routine 5 week-old cultures of P-CC, two different strains of *Pseudomonas aeruginosa* have been isolated. One of these, a facultative anaerobe growing abundantly throughout the fluid medium, was a non-Pyo producer, and the second, the Pyo producer, a strict aerobe responsible for the heavy pellicle formation and gummy material which settles to the bottom of the carboy after shaking. Thus it is important to break up the pellicle formation on the surface and to aerate the medium by agitation.

Production of Antibiotics by Pseudomonas aeruginosa

The beef extract-peptone broth which we employ as the culture medium has the following composition: Bacto-peptone (Difco) 10 gm., beef extract (Difco) 3 gm., sodium chloride (analytical reagent) 5 gm., distilled water to make 1000 ml. It is supplemented with glycerol to give a concentration of 1 per cent, as suggested by Schoental (25). The medium is adjusted to pH 7.5 to 7.8 with N sodium hydroxide solution. The medium is then placed in 12 liter Pyrex bottles (10 liters of medium per bottle) which are plugged with gauze-wrapped, non-absorbent cotton plugs, subjected to flowing steam for 1 hour, and then autoclaved at 15 pounds steam pressure for 1 hour. After sterilization, the pH of this medium is approximately 7.5.

The inoculum is prepared by transferring the organism to a flask containing 100 to 150 ml. of peptone-water medium which is then incubated at 37° for 24 hours and 5 to 10 ml. of this culture are pipetted into each bottle.

The bottles are incubated in the dark in an upright position at 37° for a period of 5 weeks. At 7 to 10 day intervals, 100 ml. of sterile glycerol are added to each bottle and the containers shaken periodically to break up the heavy pellicle and to facilitate new growth. Shorter periods of incubation (3 weeks) resulted in a lower yield, while longer periods (7 weeks or more) did not significantly increase the antibacterial activity.

The yield of active material per liter of culture fluid is approximately the same whether the bottles contain 5 or 10 liters of medium, or whether incubated in a vertical or horizontal position.

Assays

It was observed early in our study that these Pyo compounds apparently affect the metabolism of *Staphylococcus aureus* in such a manner that the production of acid is inhibited when this organism is grown on a lactose-containing medium to which the Pyo compounds have been added. Although we are unable to explain this phenomenon, it was used at the beginning of our investigation as the basis of an assay method. In this method the potency of the crude and purified materials was determined by the inhibition of lactose fermentation by *Staph. aureus* strain F. An effort was made to determine the "50 per cent acid inhibition" (determined by a bromocresol purple color standard) as an end-point. The minimal quantity of antibacterial substance which would to this extent inhibit acid production by the test organisms in 2 ml. of lactose-bromocresol purple broth[2] is referred to as the "50 per cent acid inhibition" unit. However, as it was difficult to determine this end-point accurately, a simpler method was later substituted. In this later method, suitable serial dilutions of the dissolved sample are incorporated in 1 ml. portions of the medium and inoculations are then made with an equal volume of *Staph. aureus* F culture (diluted 1:1000 in peptone-water). These tubes, including suitable controls, are incubated at 37° for 18 hours, and then inspected for visible evidence of bacterial growth. Potency of the sample is recorded as the highest dilution of the substance which prevents growth under these conditions. Accordingly, our growth inhibition unit may be defined as the minimal amount of material necessary to inhibit growth completely in 1 ml. of the assay medium. Check assays with a standard clavacin preparation carried out each day gave remarkably consistent results.

In a comparison of the two methods of assay, it was found that 1 growth inhibition unit is approximately equivalent to 2 of the "50 per cent acid inhibition" units. All assay values given in this paper are growth inhibition units unless otherwise noted.

Extraction of Antibiotics of Pseudomonas aeruginosa

After an incubation period of 5 weeks, the cultures which have become very viscous are removed from the incubator and placed in the refrigerator (5–8°) where they are allowed to remain overnight. The pH is lowered to 3.5 with dilute hydrochloric acid, which causes the viscosity to become nearly that of water, thus permitting the removal of the organisms by means of a Sharples supercentrifuge. The precipitate of the organisms is dispersed in alcohol with a Waring blendor and repeatedly extracted with hot

[2] An inoculum of 1 to 2 million organisms per ml. of medium was employed and the culture incubated at 37° for 18 hours.

95 per cent ethyl alcohol until the alcoholic extracts are colorless. The alcoholic extracts are combined and an aliquot taken for assay.

During the early part of our work the culture fluid from which the organisms had been removed was saturated with sodium chloride, acidified by adding 20 ml. of concentrated hydrochloric acid per liter, and extracted by stirring with one-fifth of its volume of butanol. The butanol was concentrated under diminished pressure and the residue examined for antibiotic potency. Owing to the low potency and low yields of active material, this extraction was discontinued.

However, before the butanol extraction was abandoned, some of these extracts were partitioned into sodium bicarbonate-soluble, carbonate-soluble, hydroxide-soluble, and neutral fractions. From the bicarbonate-soluble fraction, a yellow crystalline material was isolated. This substance sublimes at 230° and contains sulfur. Ultimate analysis and molecular weight determinations indicate the formula $C_{10}H_8NSO_3$, but because of the low antibiotic activity of this material and of the other fractions no further studies have been made.

Fractionation of Crude Antibiotic Extracts

The crude alcoholic extract of the organisms is filtered through an asbestos mat to remove the last traces of suspended material and is then diluted with distilled water to a concentration of 80 per cent alcohol and repeatedly extracted with low boiling petroleum ether. This extraction removes a large part of the neutral fats and free fatty acids which have a tendency to interfere with subsequent fractionation. Little antibiotic activity other than that due to the fatty acids themselves is removed during this extraction.

The 80 per cent alcohol solution is evaporated at room temperature and the aqueous residue is extracted exhaustively with ether. The material in this ether solution is then partitioned into four fractions as outlined in Diagram 1.

Table I illustrates the division of the total antibacterial activity in a typical crude extract.

The purification of these extracts did not appear to be promising when it was discovered that the sum of the antibiotic activities of the fractions was much less than the total activity of the unfractionated extract. Since our experience indicated a remarkable stability of the crude extracts, it seemed unlikely that this loss of potency was real. In an attempt to find the explanation for this behavior, it was observed that combinations of certain fractions were more potent than the sum of the potencies of the individual fractions. Table II gives some examples of such augmentation and shows

that recombination of the fractions of Table I in the same proportions as they occur in the crude extract accounts for all of the original potency.

Pure, crystalline, active substances have been isolated from the carbonate-soluble and the neutral fractions. These substances have been designated Pyo I, Pyo II, Pyo III, and Pyo IV, in the order of their isolation. Pyo II is obtained from the carbonate-soluble fraction and Pyo I, Pyo III, and Pyo IV are obtained from the neutral fraction.

DIAGRAM 1

Crude extract in ether solution

Extracted 5 times with 0.1 volume aqueous 5% $NaHCO_3$

- $NaHCO_3$ solution
  - Neutralized with HCl and exhaustively extracted with ether
    - Ether solution. Bicarbonate-soluble fraction. Large amount solid of low antibiotic potency
    - Aqueous solution Discarded
- Ether solution
  - Extracted 5 times with 0.1 volume aqueous 5% Na_2CO_3
    - Na_2CO_3 solution
      - Neutralized with HCl and exhaustively extracted with ether
        - Ether solution. Carbonate-soluble fraction. Solids usually have potency of 300 or more units per mg.
        - Aqueous solution Discarded
    - Ether solution
      - Extracted 5 times with 0.1 volume N NaOH
        - NaOH solution
          - Neutralized with HCl and exhaustively extracted with ether
            - Ether solution. NaOH-soluble fraction
            - Aqueous solution Discarded
        - Ether solution. Neutral fraction

In one experiment in which the bicarbonate-, carbonate-, and sodium hydroxide-soluble fractions were pooled and decolorized with charcoal, a white crystalline material was obtained. This substance melted[3] at 187° and did not contain nitrogen. Because of its low antibiotic activity and because subsequent batches of crude extracts did not yield this material, it has not been further investigated.

Bicarbonate-Soluble Fraction—From the bicarbonate-soluble fraction a

[3] The Fisher-Johns apparatus was used to determine all melting points reported in this paper.

yellow crystalline material which sublimes at 220° has been isolated. It has a characteristic ultraviolet absorption spectrum and its ultimate analysis and molecular weight fit the formula $C_{14}H_{10}N_2O_2$. Because of its very low antibiotic activity this compound has not been investigated further.

Carbonate-Soluble Fraction; Isolation of Pyo II—The carbonate-soluble fraction is dissolved in benzene and passed through a column (10 × 1 cm.) of dried oxalic acid. The activity remains on the column, whereas most of the solids and brown color are not retained. After thorough washing with benzene, the oxalic acid and adsorbed material are dissolved in a saturated solution of potassium bicarbonate and ether. The ether is then extracted

TABLE I

Partition of Typical Crude Extract

Fraction	Nature of material	Weight	Per cent of total weight	Activity	Total growth inhibition units in fraction
		gm.		*growth inhibition units per mg.*	
A	Bicarbonate-soluble	55.41	43.2	160	8,800,000
B	Carbonate-soluble	7.78	6.1	2560	19,900,000
C	NaOH-soluble	2.06	1.6	2560	5,300,000
D	Neutral	21.08	16.4	1280	27,000,000
E	Petroleum ether-soluble	41.82	32.6	40	1,700,000
Total		128.15	99.9		62,700,000

The crude extract which was one of our best preparations contained 128.15 gm. of solids having a total potency of approximately 186,000,000 growth inhibition units; it was obtained from the organisms of 1250 liters of culture media. Approximately 97 gm. (Fractions A and E) did not contain the specific antibiotics of *Pseudomonas aeruginosa* and were discarded. 390 mg. of pure Pyo II were obtained from Fraction B. Although Fraction D has not been processed, other preparations of similar character have given 50 to 100 mg. of pure Pyo III, and 250 to 400 mg. of a mixture of Pyo Ib and Pyo Ic per gm. of solids.

four times with saturated potassium bicarbonate to remove the last traces of oxalic acid. The active material is then removed from the ether by six extractions with 5 per cent sodium carbonate and again transferred to ether by extraction of the aqueous phase after acidification to pH 4 to 5 with hydrochloric acid. After being washed with water, the ether is distilled and the residue containing the active material is crystallized from benzene. The crystals are dissolved in alcohol, decolorized with charcoal, and recrystallized from acetone, methanol, and finally from a small amount of

ethyl alcohol. This procedure yields a small amount of slightly yellow colored scales which melt at 149°.

While the above procedure gives the best results, crystalline Pyo II may also be obtained by the following method: The carbonate-soluble fraction is dissolved in the minimal amount of alcohol and poured into 10 volumes of N sodium hydroxide. This alkaline solution is chilled and the soaps (largely sodium palmitate) are removed by filtration. This material is re-precipitated by dissolving in warm N sodium hydroxide and repeating the

TABLE II

Augmentation Studies of Fractions Shown in Table I

Tube No.	Fraction A, bicarbonate-soluble	Fraction B, carbonate-soluble	Fraction C, sodium hydroxide-soluble	Fraction D, neutral	Fraction E, petroleum ether-soluble	Growth inhibition units per mg.	
						By assay	By calculation
	mg. per ml.	*mg. per ml.*	*mg. per ml.*	*mg. per ml.*	*mg. per ml.*		
1	1.0					>160, <320	
2		1.0				>2560, <5120	
3			1.0			>2560, <5120	
4				1.0		>1280, <2560	
5					1.0	>40, <80	
6	0.88	0.12				>320, <640	>448, <896
7	0.96		0.04			>320, <640	>256, <512
8	0.72			0.28		>1280, <2560	>473, <946
9	0.56				0.44	>40, <80	>107, <214
10		0.80	0.20			>2560, <5120	>2560, <5120
11		0.28		0.72		>2560, <5120	>1638, <3276
12		0.16			0.84	>320, <640	>443, <886
13			0.10	0.90		>2560, <5120	>1408, <2816
14			0.04		0.96	>40, <80	>140, <280
15				0.34	0.66	>160, <320	>461, <922
16	0.44	0.06	0.02	0.16	0.32	>1280, <2560	>492, <984
17	Tube 16, 1:3					>1920, <3840	>492, <984
18	" 16, 1:5					>1600, <3200	>492, <984

From Tubes 16, 17, and 18, the activity of a mixture of Fractions A, B, C, D, and E (mixed in proportion to their occurrence in the original extract) is seen to be about 1920 growth inhibition units per mg. From this value the total activity of the crude extract can be calculated to be 246,000,000 units.

chilling process. The combined sodium hydroxide filtrates are treated with a slight excess of barium hydroxide and the last traces of fatty acids removed by filtering off their barium soaps. The alkaline filtrate is then acidified with hydrochloric acid to pH 4 to 5 and the active material is recovered by ether extraction. The crystalline product is then obtained in the manner described above. Although Pyo II was isolated in this manner over 2 years ago, this method has been abandoned in favor of the shorter and more efficient procedure.

Sodium Hydroxide-Soluble Fraction—From this fraction an additional amount of Pyo II may be obtained. The balance of the solids appears spectroscopically to consist of a mixture of compounds similar to that found in the neutral fraction. Repartition of this fraction results in only a small amount of solid remaining in the sodium hydroxide-soluble fraction.

Neutral Fraction; Preparation and Purification of Pyo I, Pyo III, and Pyo IV—The neutral fraction, which is obtained as a brown oily residue, is dissolved in chloroform and poured on a column (20 × 5 cm.) of permutit which has been washed previously with chloroform. The column is washed with chloroform and appropriate fractions are collected. The first two or three fractions contain a brown oil which accounts for 25 to 50 per cent of the weight of the neutral fraction but continued washing removed only small additional amounts of material. The release of the adsorbed material is then accelerated by the addition of small amounts of absolute ethyl alcohol to the chloroform. Successive fractions thus obtained from the column are studied spectroscopically[4] to ascertain the homogeneity of the eluted material. It was found that three characteristic absorption curves, later associated with the purified preparations of Pyo I, Pyo III, and Pyo IV, could be distinguished in the absorption curves of such fractions. By determination of the $E_{1\,\text{cm.}}^{1\%}$ values of these fractions at the points of maximal absorption of pure Pyo I, Pyo III, and Pyo IV, the percentage of each can be approximated. (Fig. 1 shows the ultraviolet absorption spectra of successive fractions obtained from a typical column.) By this means it was found that first Pyo IV, then Pyo III, and finally Pyo I are eluted, while the pyocyanine remains on the permutit and can be removed only by washing with ethyl alcohol. An example of the purification with a permutit column is given in Table III.

Preparation of Pyo IV—As mentioned above, Pyo IV occurs in the first few fractions from the permutit column; it is contaminated with a large amount of reddish oil. The separation from this oily material is effected by adsorbing the mixture on a permutit column and fractional elution with low boiling petroleum ether containing a small proportion of ethanol. The red, oily components pass through the permutit rapidly, while the Pyo IV is removed more slowly. Alumina columns may be used in the same manner to effect this separation. The solid fractions shown spectroscopically to contain Pyo IV are pooled and crystallized first from a 1:1 mixture of methanol and low boiling petroleum ether, then from acetone, and finally from ethanol. Pyo IV is a colorless, crystalline (needles) substance which appears to have two allotropic forms; one melts at 131–132° and the other at 140°.

[4] A Beckman quartz spectrophotometer was used in the spectroscopic work reported in this paper.

TABLE III

Partition of Neutral Fractions by Means of Permutit Column

A crude neutral fraction which weighed 10.48 gm. was dissolved in chloroform and poured on a column (20 × 5 cm.) of permutit which had been washed previously with chloroform.

Fraction No.	Volume of solvent	Solvent used	Weight of fraction	Per cent Pyo III in Pyo I
	ml.		*mg.*	
1	1000	Chloroform	2436*	
2	500	"	106.7*	
3	500	0.5% ethyl alcohol in $CHCl_3$	17.1	12
4	200	0.5% " " " "	70.7	15
5	200	0.5% " " " "	736	30
6	200	0.5% " " " "	876	35
7	200	0.5% " " " "	768	15
8	200	0.5% " " " "	392	12
9	200	0.5% " " " "	321	12
10	200	0.5% " " " "	232	12
11	200	0.5% " " " "	252	12
12	200	0.5% " " " "	388	10
13	200	0.5% " " " "	254	10
14	200	0.5% " " " "	320	10
15	200	0.5% " " " "	389	6
16	200	0.5% " " " "	140	5
17	200	0.5% " " " "	414	5
18	200	0.5% " " " "	303	5
19	200	0.5% " " " "	157	5
20	200	0.5% " " " "	140	3
21	200	0.5% " " " "	80.2	2
22	200	0.5% " " " "	67.9	2
23	200	0.5% " " " "	51.2	
24	200	0.5% " " " "	47.0	
25	200	0.5% " " " "	76.8	2
26	200	0.5% " " " "	63.9	
27	200	0.5% " " " "	59.6	
28	200	1% ethyl alcohol in $CHCl_3$	53.6	Trace
29	200	1% " " " "	36.2	
30	200	1% " " " "	46.7	
31	200	1% " " " "	42.4	Trace
32	400	1% " " " "	47.1	
33	400	1% " " " "	38.1	Crude Pyo I, no Pyo III
34	400	2% " " " "	17.3	
35	200	2% " " " "	11.6	
36	200	2% " " " "	13.3	
37	200	2% " " " "	8.7	
38	400	5% " " " "	18.1†	
39	200	95% " "	36.5†	
40	400	95% " "	67.6†	

* Brown oil.

† Purple color.

Preparation of Pyo III—Fractions containing about 15 per cent Pyo III (such as Fractions 4, 7, and 8 in Table III) were combined and passed through another column of permutit. The data are given in Table IV.

The fractionation of the 1.23 gm. of material that contained 15 per cent Pyo III described in Table IV yielded 424 mg. (Fractions e to l) which contained 55 to 60 per cent Pyo III and 602 mg. (Fractions n to r) which contained 10 per cent or less Pyo III. Judging by the ultraviolet absorption spectra, the main component of Fractions n to r is Pyo I. (For purification see a subsequent section.)

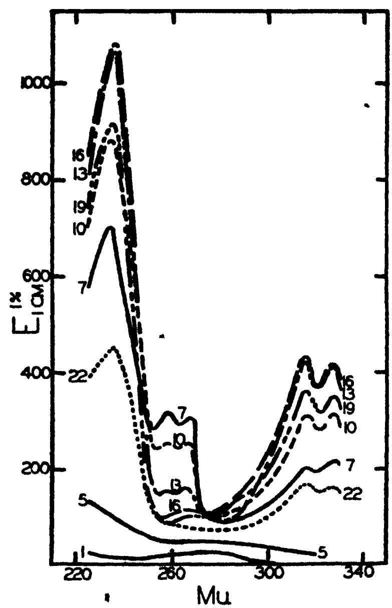


FIG. 1. Ultraviolet absorption spectra of successive fractions obtained from a permutit column. The curve number is the fraction number.

Fractions from the columns containing 25 or more per cent of Pyo III (such as combined Fractions 5 and 6 from Table III) yielded Pyo III after repeated crystallization from mixtures of ethanol and acetone, methanol, benzene, and 50 per cent ethanol. After decolorization with norit A and crystallization from methanol, the final, colorless, crystalline (rectangular prisms) product melts at 152.8–153.5°.

Preparation of Pyo Ib and Pyo Ic—Although Pyo I was obtained in crystalline form over 2½ years ago, there was considerable uncertainty regarding its purity. Usually this material was obtained as beautiful

colorless cystals having a melting point of 129–130°. Repeated crystallizations of this product from a number of solvents did not raise the melting point. However, by adsorption of this material on a long (180 × 2.5 cm.) column of permutit and fractional elution with petroleum ether containing a small proportion of absolute ethyl alcohol, some material was recovered which melted at a higher temperature. If the higher melting fractions were combined and again passed, in the same manner, through a permutit

TABLE IV

Concentration of Pyo III by Means of Permutit Column

1.23 gm. containing about 15 per cent Pyo III in Pyo I were dissolved in chloroform and poured on a column (20 × 5 cm.) of permutit which had been washed with chloroform.

Fraction	Volume of solvent	Solvent used	Weight of fraction	Per cent Pyo III in Pyo I
	ml.		*mg.*	
a	600	Chloroform	10.3*	
b	1300	0.25% ethyl alcohol in $CHCl_3$	9.4*	
c	500	0.25% " " " "	5.5*	
d	500	0.25% " " " "	11.7	4
e	100	0.25% " " " "	7.1	50
f	100	0.25% " " " "	16.6	70
g	100	0.25% " " " "	24.9	70
h	100	0.25% " " " "	37.0	70
i	100	0.25% " " " "	53.0	
j	100	0.25% " " " "	80.5	65
k	100	0.25% " " " "	84.0	
l	100	0.25% " " " "	119.1	50
m	100	0.25% " " " "	106.8	15
n	100	0.25% " " " "	82.1	10
o	100	0.25% " " " "	57.3	10
p	100	0.25% " " " "	46.8	10
q	100	0.25% " " " "	43.0	
r	100	0.25% " " " "	116.5	7

* Very crude.

column, a small amount of a colorless crystalline product which melted at 142–143° was obtained.

In an effort to facilitate the fractionation of the material melting at 129–130° into its component parts, the following empirical procedure was evolved. The Pyo I fraction, freed of Pyo III as described above, is dissolved in alcohol-free diethyl ether to give a solution having a concentration of 1 mg. per ml. and this solution is extracted between 80 and 100 times with one-tenth of its volume of either N potassium hydroxide or 0.1 N hydro-

chloric acid. The ether solution is then shaken with N hydrochloric acid and allowed to stand overnight in the refrigerator in contact with the acid. A crystalline hydrochloride which appears in both the ether and acid layers is removed by filtration. This solid is dissolved in ethyl alcohol, 100 volumes of ether added, and the solution is extracted with 5 per cent sodium bicarbonate to remove the acid, then washed with water, and distilled. The residue, after crystallization from 50 per cent aqueous alcohol, decolorization in alcoholic solution with charcoal, and recrystallization from aqueous alcohol, yields a colorless crystalline solid which melts at 139°. This material, Pyo Ic, has an antibiotic activity of 1280 units per mg. Pyo Ic cannot be further fractionated by means of a permutit column.

The combined N potassium hydroxide (or 0.1 N hydrochloric acid) extracts are neutralized and the precipitate extracted with ether. The ether, after being washed with water, is distilled and the residue is dissolved in sufficient alcohol-free ether to give a concentration of 1 mg. per ml. This solution is extracted 90 times with one-tenth of its volume of 0.1 N hydrochloric acid. The material is recovered from the combined acid washings by extraction with ether after adjustment of the pH to 5 to 7. This process of dissolving the material in ether and extracting the ether solution with 0.1 N hydrochloric acid is repeated until 95 to 100 per cent of the material dissolved in the ether is extracted by the acid. The product obtained from the final hydrochloric acid extract is decolorized in alcoholic solution with norit A and crystallized from acetone. This compound is designated Pyo Ib.

An alternative method of purification of Pyo Ib which has been extensively used is fractionation by adsorption on a column (120 × 12 cm.) of permutit. The fraction of Pyo Ib relatively free from Pyo Ic is dissolved in a small volume (15 to 25 ml.) of chloroform and poured on a column of permutit which has been washed previously with chloroform. The Pyo Ib is eluted from the column with petroleum ether (b.p. 70–100°) containing 2 per cent absolute ethyl alcohol, and as the elution progresses increasing proportions up to 5 per cent of ethyl alcohol are added. The first fractions have melting points in the range 100–130° but most of the solid is recovered in fractions which have melting points from 140–144°. Repetition of the purification by passage through the permutit may be desirable to obtain pure Pyo Ib. In one case the passage was repeated several times but the product was not improved by the last passage. The fractions of highest melting point were combined and crystallized from 30 to 40 per cent ethyl alcohol and then from acetone. This compound is a colorless, crystalline substance which melts at 146.2–147°.

Diagram 2 summarizes the separation of the neutral fraction into the various Pyo compounds.

Physical and Chemical Properties of Pyo Compounds

Pyo Ib and Ic—The physical properties of these two substances are very similar. The melting points, ultimate analyses, molecular weights, and

DIAGRAM 2

Crude neutral fraction

Dissolve in $CHCl_3$ and pour on permutit column

Red oil←

Very crude Pyo IV←

Pass through permutit column in low-boiling petroleum ether to give Pyo IV

Start 0.25% absolute alcohol

40–50% Pyo III in Pyo I (mixture)

Crystallize from acetone-ethyl alcohol solution

10–20% Pyo III in Pyo I (mixture)

70–80% Pyo III in Pyo I (mixture)

Pass through permutit column

Repeat crystallization

→Pure Pyo III

40–50% Pyo III in Pyo I (mixture)

Crystallize as above, etc.

10–20% Pyo III in Pyo I (mixture)

Repeat column treatment, etc.

3–8% Pyo III in Pyo I (mixture)

1% absolute alcohol in $CHCl_3$

Hydrogenate in alcohol

Pyo I. Dissolve in ether

95% ethyl alcohol-

Exhaustively extract with N KOH or 0.1 N HCl

Pyocyanine←

N KOH or 0.1 N HCl. Neutralize; extract with ether, repeat N KOH extraction

Ether. Ppt. as hydrochloride; decompose; crystallize. Yields Pyo Ic.

N KOH. Neutralize and recover solids; crystallize or pass through permutit column. Yields Pyo Ib.

antibiotic activities of these two substances and of their hydrogenated products are given in Table V. They are extremely soluble in the various alcohols, readily soluble in $CHCl_3$, benzene, and dioxane, and may be crystallized from acetone or ether. However, it has not been possible to separate a mixture of the two into its component parts by crystallization procedures. If pure, either of these two compounds will depress the melting point of the other.

These two compounds are characterized by their unusual stability and the inertness of their oxygen and nitrogen atoms. All attempts to prepare derivatives of hydroxyl, carbonyl, amide, and primary, secondary, or tertiary amines have been unsuccessful with a mixture of Pyo Ib and Pyo Ic. These compounds are precipitated from ether solution by oxalic acid, flavianic acid, and hydrochloric acid, as described above, but since these salts tend to decompose during purification they have not been satisfactory for analytical purposes. A mixture of these compounds remains unchanged even after standing in concentrated sulfuric acid on the boiling water bath overnight and can be recovered by simply diluting the acid with water, neutralizing with $NaHCO_3$, and extracting the aqueous phase with ether. A mixture of Pyo Ib and Pyo Ic does not form a methiodide under a variety of conditions. Treatment of samples of Pyo Ib and Pyo Ic with sodium hydroxide and iodine yields new compounds having antibiotic activities of 1280 and 10,000 units per mg., respectively. The determination of the iodine number by the method of Yasuda (31) gave a value of 94 for a sample of Pyo Ic. From this value, the molecular weight of 540 can be calculated, assuming the addition of 2 moles of halogen. Pyo Ib and Pyo Ic do not hydrogenate in alcoholic solution when the Adams platinum oxide catalyst is used, but in glacial acetic acid, in the presence of the same catalyst, both compounds take up 4 moles of hydrogen per mole of material. The colorless product obtained by hydrogenating Pyo Ib in glacial acetic acid melts at 164°. Pyo Ic hydrogenated in a similar manner yields a compound which melts at 157°. The octahydro Pyo Ib depresses the melting point of the octahydro Pyo Ic.

Pyo II—Pure Pyo II crystallizes in light yellow platelets from ethyl alcohol. Its ultimate analysis, molecular weight, melting point, and antibiotic activity are given in Table V. This substance is sparingly soluble in cold acetone, benzene, and methyl alcohol, is insoluble in petroleum ether, water, and 5 per cent sodium bicarbonate, but dissolves readily in aqueous carbonate solution. An alkaline solution of Pyo II possesses a yellow color which disappears upon acidification. Pyo II does not give a positive test with ferric chloride, does not form a silver salt in alcoholic solution or a barium salt from aqueous alkaline solution, and all attempts to prepare a S-benzylthiuronium salt, or a *p*-nitrobenzyl ester or *p*-bromophenacyl ester were unsuccessful. The substance reacts with diazomethane in dry diethyl ether to yield an oil. Hydrogenation in glacial acetic acid in the presence of Adams' platinum oxide catalyst results in an uptake of 4 moles of hydrogen per mole of Pyo II. The hydrogenated product crystallizes as fine, colorless needles which melt at 109°.

Pyo III—Pyo III is a colorless crystalline solid. The melting point,

ultimate analysis, molecular weight, and antibiotic activity of this substance are listed in Table V. It has essentially the same solubility as Pyo Ib and Pyo Ic, being in general less soluble than the latter, particularly in dioxane.

TABLE V

*Analyses of Pyo Compounds and Their Hydrogenated Products**

Compound	Formula	Theory				Found				Activity	M.p.
		C	H	N	Mol. wt.	C	H	N	Mol. wt.†		
		per cent	*per cent*	*per cent*		*per cent*	*per cent*	*per cent*		*growth inhibition units per mg.*	°C.
Pyo Ib	$C_{31}H_{40}N_2O_2$	78.77	8.53	5.93	472.6	78.55	8.6	5.7	440	160	146.2–147
						78.8	8.7				
" Ic‡	$C_{34}H_{48}N_2O_2$	79.02	9.36	5.42	516.7	79.4	9.41	5.35	486	1,280	138.8–139.2
						79.42	9.48	5.3			
" II	$C_{34}H_{46}N_2O_4$	74.69	8.48	5.12	546.7	74.60	8.52	5.2	546	10,000	149 –149.5
						74.49	8.93	5.1			
" III	$C_{34}H_{44}N_2O_2$	79.64	8.65	5.46	512.7	79.54	8.8	5.5	502	300	152.8–153.5
" IV	$C_{16}H_{23}NO_3$	69.28	8.36	5.05	277.4	69.45	8.65	5.05	280	120	131 – 2
						69.58	8.65		285	120	139.5–140
Tetrahydro Pyo III‡	$C_{34}H_{48}N_2O_2$	79.02	9.36	5.42	516.7	79.28	9.24	5.36	518	1,280	138.8–139.2
Octahydro Pyo Ib	$C_{31}H_{48}N_2O_2$	77.45	10.07	5.83	480.7	77.53	10.00	5.42	518	40	163.8–164
Octahydro Pyo Ic§	$C_{34}H_{56}N_2O_2$	77.81	10.76	5.34	524.8	78.14	10.86	5.8	554	40	156.8–157
Octahydro Pyo II	$C_{34}H_{54}N_2O_4$	73.60	9.81	5.05	554.8	73.46	9.90	5.1	535	640	109 –109.2
Dodecahydro Pyo III§	$C_{34}H_{56}N_2O_2$	77.81	10.76	5.34	524.8	77.80	10.41	5.6	542	40	156.8–157
Pyo IV dibenzoate	$C_{30}H_{34}NO_5$	73.75	7.02	2.87	488.6	74.11	6.97	2.70	487		101.9–102

* Considerable difficulty was encountered in the purification of the Pyo compounds and especially in their analyses. Although the empirical formulas listed fit our analytical data best, it is recognized that the true formulas may differ by as much as a methylene group.

† Rast method. Tribromophenol was used as solvent.

‡ Identical spectra and antibiotic activity. No depression of mixed melting points.

§ Identical spectra and antibiotic activity. No depression of mixed melting points.

Although our evidence indicates that Pyo III is somewhat less stable than Pyo Ic, all attempts to prepare an acetate, oxime, and a semicarbazone have failed. Determination of the iodine number by the method of Yasuda (31) gives an average value of 146, indicating a molecular weight of 522, assuming that 3 moles of halogen are added per mole. Pyo III takes up 2

moles of hydrogen per mole when it is hydrogenated in ethyl alcohol in the presence of Adams' platinum oxide catalyst and in glacial acetic acid 6 moles are added. The tetrahydro Pyo III melts at 139° and the melting point is not depressed by Pyo Ic but is by Pyo Ib. The tetrahydro Pyo III is identical with Pyo Ic as regards analysis and ultraviolet absorption spectrum, and both have an antibiotic activity of 1280 units per mg. The dodecahydro Pyo III melts at 157° and is identical with octahydro Pyo Ic with respect to melting point (no depression when mixed), ultimate analysis, and antibiotic activity. These data are shown in Table V.

Because of the difficulty of removing the last traces of Pyo III from Pyo I, we have recently utilized the conversion of Pyo III to Pyo Ic by hydrogenation to obtain Pyo I preparations free of Pyo III. The crude Pyo I fraction is hydrogenated in alcoholic solution at 45 pounds pressure with Adams' platinum oxide catalyst and then the Pyo III-free material is separated into Pyo Ib and Pyo Ic as previously described.

Pyo IV—Pure Pyo IV crystallizes in the form of colorless needles. Its melting point, composition, molecular weight, and antibiotic activity are listed in Table V.

Since Pyo IV took up no halogen under the conditions of Yasuda's (31) method for the determination of iodine numbers, hydrogenation of this compound in alcohol was not attempted. However, in glacial acetic acid solution and in the presence of Adams' platinum oxide catalyst Pyo IV adds 2 moles of hydrogen per mole. It forms a dibenzoate which melts at 102° (see Table V for the analyses and molecular weights). Pyo IV also forms a 2,4-dinitrophenylhydrazone on treatment of an alcoholic solution with Brady's reagent (32).

Ultraviolet Absorption Spectra

Pyo Ib and Pyo Ic—Fig. 2 shows the ultraviolet absorption spectra of these compounds. Fig. 3 shows the curves of octahydro Pyo Ib and octahydro Pyo Ic.

If Pyo Ib is dissolved in 0.016 N alcoholic potassium hydroxide, the 2360 A maximum is shifted to 2430 A with a decrease in $E^{1\%}_{1\,\mathrm{cm.}}$ value (to 1025) and the 3160 and 3280 A maxima merge into a single peak at 3150 A ($E^{1\%}_{1\,\mathrm{cm}}$ 350). Dissolving Pyo Ib in 0.01 N alcoholic hydrochloric acid causes the 2360 A peak to shift to 2320 A and the $E^{1\%}_{1\,\mathrm{cm.}}$ value to increase to 1760, while the maxima at 3160 and 3280 A merge into a single peak at 3000 A ($E^{1\%}_{1\,\mathrm{cm.}}$ 280).

If Pyo Ic is dissolved in 0.016 N alcoholic potassium hydroxide, there is a change in its ultraviolet absorption spectrum. A new maximum at 2420 A ($E^{1\%}_{1\,\mathrm{cm.}}$ 976) replaces the 2360 A maximum of Pyo Ic, and at the same

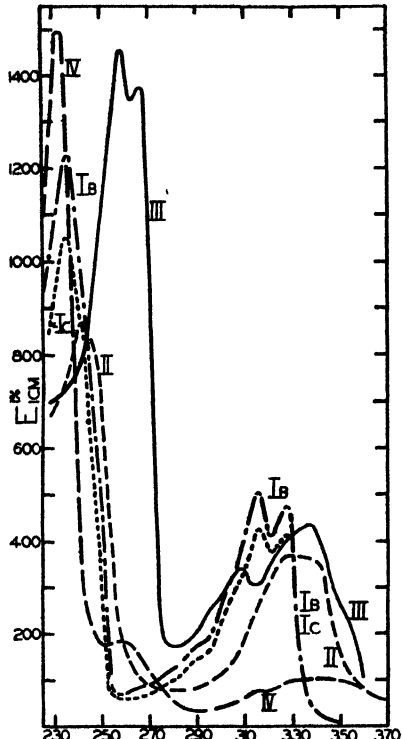


FIG. 2

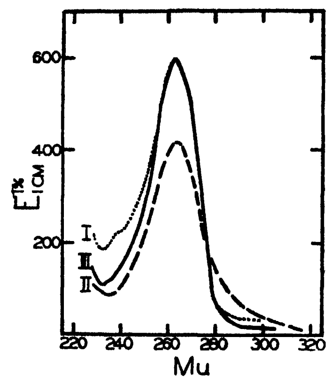


FIG. 3

FIG. 2. Curve Ib, Pyo Ib, maxima 2360 A ($E^{1\%}_{1\,\mathrm{cm.}}$ 1225), 3160 A ($E^{1\%}_{1\,\mathrm{cm.}}$ 505), and 3280 A ($E^{1\%}_{1\,\mathrm{cm.}}$ 470); minima 2600 A ($E^{1\%}_{1\,\mathrm{cm.}}$ 65) and 3220 A ($E^{1\%}_{1\,\mathrm{cm.}}$ 415). Curve Ic, Pyo Ic, maxima 2360 A ($E^{1\%}_{1\,\mathrm{cm.}}$ 1050), 3160 A ($E^{1\%}_{1\,\mathrm{cm.}}$ 431), and 3280 A ($E^{1\%}_{1\,\mathrm{cm.}}$ 415); minima 2600 A ($E^{1\%}_{1\,\mathrm{cm.}}$ 53) and 3220 A ($E^{1\%}_{1\,\mathrm{cm.}}$ 383). Curve II, Pyo II, maxima 2420 A ($E^{1\%}_{1\,\mathrm{cm.}}$ 873) and 3300 A ($E^{1\%}_{1\,\mathrm{cm.}}$ 369); minimum 2860 A ($E^{1\%}_{1\,\mathrm{cm.}}$ 77). Curve III, Pyo III, maxima 2580 A ($E^{1\%}_{1\,\mathrm{cm.}}$ 1460), 2660 A ($E^{1\%}_{1\,\mathrm{cm.}}$ 1376), 3070 A ($E^{1\%}_{1\,\mathrm{cm.}}$ 340), and 3380 A ($E^{1\%}_{1\,\mathrm{cm.}}$ 434); minima 2630 A ($E^{1\%}_{1\,\mathrm{cm.}}$ 1350), 2800 A ($E^{1\%}_{1\,\mathrm{cm.}}$ 172), and 3150 A ($E^{1\%}_{1\,\mathrm{cm.}}$ 304). Curve IV, Pyo IV, maxima 2320 A ($E^{1\%}_{1\,\mathrm{cm.}}$ 1480), 2580 A ($E^{1\%}_{1\,\mathrm{cm.}}$ 183), and 3400 A ($E^{1\%}_{1\,\mathrm{cm.}}$ 103); minima 2500 A ($E^{1\%}_{1\,\mathrm{cm.}}$ 174) and 2900 A ($E^{1\%}_{1\,\mathrm{cm.}}$ 35). Ethyl alcohol (95 per cent) was used as the solvent in each case.

FIG. 3. Curve I, octahydro Pyo Ib, maximum 2640 A ($E^{1\%}_{1\,\mathrm{cm.}}$ 580); minimum 2320 A ($E^{1\%}_{1\,\mathrm{cm.}}$ 185). Curve II, octahydro Pyo II, maximum 2630 A ($E^{1\%}_{1\,\mathrm{cm.}}$ 415); minimum 2360 A ($E^{1\%}_{1\,\mathrm{cm.}}$ 85). Curve III, dodecahydro Pyo III and octahydro Pyo Ic, maximum 2640 A ($E^{1\%}_{1\,\mathrm{cm.}}$ 580); minimum 2320 A ($E^{1\%}_{1\,\mathrm{cm.}}$ 100). Ethyl alcohol (95 per cent) was used as the solvent in each case.

time the maxima at 3160 and 3280 A merge into one maximum at 3150 A ($E^{1\%}_{1\,\mathrm{cm.}}$ 326). The absorption spectrum of a 0.01 N alcoholic hydrochloric

acid solution of Pyo Ic behaves in a manner similar to that of Pyo Ib with maxima at 2320 A ($E^{1\%}_{1\,cm.}$ 1880) and 3000 A ($E^{1\%}_{1\,cm}$ 316) replacing those at 2360, 3160, and 3280 A.

Pyo II—Fig. 2 shows the ultraviolet absorption spectrum of this compound. Fig. 3 gives the curve for the octahydro Pyo II.

Pyo III—Fig. 2 shows the ultraviolet absorption spectrum for this substance. Pyo III dissolved in hexane has an absorption spectrum somewhat different from that in alcohol in that it exhibits maxima at 2580 and 3380 A only ($E^{1\%}_{1\,cm.}$ 1175 and 340, respectively) and minima at 2430 and 2840 A ($E^{1\%}_{1\,cm.}$ 985 and 140, respectively) with shoulders at 2500, 2660, and 3100 A. If Pyo III is dissolved in 0.016 N alcoholic potassium hydroxide, the maxima at 2580 and 2660 A merge into a single peak at 2600 A with an increased $E^{1\%}_{1\,cm.}$ value (2170) and the two peaks at 3070 and 3380 A likewise become a single peak at 3240 A ($E^{1\%}_{1\,cm.}$ 550). Acidification of the alkaline solution after it has stood overnight fails to restore the original absorption curve. The absorption spectrum of a solution of Pyo III in alcohol containing 0.01 mole of hydrochloric acid per liter shows a general decrease in the $E^{1\%}_{1\,cm.}$ values but the shape of the curve remains the same. Pyo III in alcohol solution (10 γ per ml.) exhibits changes in its absorption of ultraviolet light after the solution has stood from 30 to 60 days at room temperature either in the daylight or in the dark. The cause of this change has not been investigated. The ultraviolet absorption curve of tetrahydro Pyo III is identical with that of Pyo Ic (see Fig. 2). The dodecahydro Pyo III in alcoholic solution has an absorption curve which exhibits a maximum at 2640 A ($E^{1\%}_{1\,cm.}$ 580) and a minimum at 2320 A ($E^{1\%}_{1\,cm.}$ 100). Fig. 3 shows this curve which is identical with the ultraviolet absorption curve of octahydro Pyo Ic.

Pyo IV—In alcoholic solution, Pyo IV has the ultraviolet absorption curve shown in Fig. 2. If Pyo IV is dissolved in 0.016 N alcoholic potassium hydroxide, its maxima are shifted at 2440 A ($E^{1\%}_{1\,cm.}$ 1270) and 2740 A ($E^{1\%}_{1\,cm.}$ 240); 0.01 N alcoholic hydrochloric acid has no effect upon the ultraviolet absorption spectrum of this compound.

The hydrogenation studies and ultraviolet absorption spectra show that Pyo Ib, Pyo Ic, Pyo II, and Pyo III are related structurally. This is also indicated by the stability of the compounds with respect to the inertness of the nitrogen and oxygen atoms present in the common nucleus.

Bacteriological Properties

The cultural studies which have been undertaken have dealt for the most part with crude alcoholic extracts but in some experiments a crystalline compound (Pyo II) has been employed. This crystalline product contains

from 10,000 to 20,000 units per mg. and is, therefore, active in a dilution of 1:10 to 20 million.

The activities of a crude extract and of the crystalline product have been studied in relation to several types of bacteria. The liquid medium employed in these tests was that used in the original routine acid inhibition assay procedure. In certain instances, this medium was supplemented with 1 per cent horse serum to support the growth of the test organism. Table VI compares the degree of activity observed with different test or-

TABLE VI

Antibacterial Activity of Crude Pyo Preparation and of Pure Pyo II against Various Gram-Positive and Gram-Negative Organisms

The preparation was diluted serially in 1 ml. of lactose-bromocresol purple broth and the following organisms (4,000,000) added in 1 ml. of the same broth. The antibacterial activity is given as the number of units of the preparation required to inhibit the organisms over a period of 18 hours at 37°.

Organisms tested		No. of "50 per cent acid inhibition" units	
		Crude preparation	Pyo II
Gram-positive	*Staphylococcus aureus* F	1	1.0
	" " "*	4	2
	" " (Wood 46)	0.125	4.0
	Streptococcus hemolyticus 10-8*	2.0	64
	Corynebacterium diphtheriae 19	0.125	4.0
	" *hoffmannii**		1.0
	" *xerose*	0.125	256
	*Streptococcus viridans**	32	256
	Bacillus anthracis	1.0	8
	" " *	1.0	
	" *subtilis*	0.5	4
	Diplococcus pneumoniae Goodner 1*	4.0	64.0
Gram-negative	*Neisseria catarrhalis* m	8.0	256
	*Brucella suis**	32.0	128
	" *abortus*	16.0	64
	" " *		256
	" *suis*	16.0	128
	" *melitensis**	32	256
	Eberthella typhosa 901-0	16.0	256
	Salmonella enteriditis 52	16.0	256
	Proteus vulgaris 10	16.0	256
	Escherichia coli 1	16.0	256
	Serratia marcescens	16.0	256
	Yeast	16	256

* Diluted in the presence of 1 per cent serum.

ganisms. These results indicate that the active substances produced by *Pseudomonas aeruginosa* are highly active against the Gram-positive and only slightly active against Gram-negative bacteria.

Experiments in which a crude extract was tested against a susceptible Gram-positive organism (*Staphylococcus aurcus*) indicate that bactericidal activity in higher concentrations and bacteriostatic effects in lower concentrations are displayed. Table VII gives a typical protocol in which the number of viable bacteria in each tube of a routine assay was determined at intervals subsequent to inoculation. The results indicate that in an effective dilution (*e.g.*, 1:80) there is an initial decrease in the number of organisms during the first 2 hours. Following this decrease, the number

TABLE VII

*Mode of Action of Crude Pyo Extract**

Time	Dilution							Control
	20	40	80	160	320	640	1280	
	Viable bacteria per ml.							
15 min.	605,000	1,500,000	980,000	1,105,000	1,125,000	1,275,000	871,000	1,108,000
2 hrs.	1,340	295,000	563,000	836,000	3,820,000	5,750,000	14,200,000	22,600,000
4 "	1,650	15,750	505,000	1,000,000	5,780,000	8,080,000	21,250,000	2,450,000,000
8 "	450	18,300	429,000	1,096,000	5,860,000	710,000,000	381,500,000	
	Acid production in lactose broth							
8 hrs.	–	–	–	–	–	–	–	+++
18 "	–	–	–	–	++	+++	++++	++++

* 1,170,000 organisms (*Staphylococcus aureus*) per ml. were used as the inoculum. A partially purified extract of mixed antibiotics from *Pseudomonas aeruginosa* was used for these experiments.

remains more or less constant for the duration of the experiment. In dilutions above the effective range there is a slow increase in the number of organisms which however never approaches the control during the first 8 hours. Visible growth (*i.e.*, 1 million or more cells per ml.) does not appear in the lower dilutions even after 3 days. The effect upon acid production under these conditions is also shown in Table VII.

While the mode of action of these substances is not known, experiments on semisynthetic and completely synthetic media indicate that the carbohydrate, peptone, and other complex nitrogenous components of the lactose-bromocresol purple broth do not alter the activity of these compounds. Added *p*-aminobenzoic acid and nicotinic acid amide are also without effect, while riboflavin appears to enhance the antibiotic activity to a slight extent.

Serum reduces but does not eliminate the effectiveness of the extracts and of the crystalline Pyo II. Furthermore, the extracts appear to be equally active against *Staphylococcus aureus* under anaerobic and aerobic conditions but are on the other hand only slightly active against the obligatory anaerobes.

The activities of Pyo Ic, Pyo II, and crystalline sodium penicillin G were compared against several test organisms by the serial dilution method. Table VIII summarizes these studies.

TABLE VIII

Antibacterial Action of Penicillin (Na Salt) and Crystalline Pyo Preparations

Dilution in which visible growth is inhibited. The concentration of crystalline materials equals 1.0 mg. per ml.

Organism	Pyo Ic	Pyo II	Penicillin Na
Bacillus subtilis	<20	1280	<20
Corynebacterium xerose	320	320	20,480
Staphylococcus aureus F	320	2560	40,960
Escherichia coli	<20	<20	<20
*Streptococcus hemolyticus**	40	80	>163,840
Bacillus anthracis		1280	>81,920
Neisseria catarrhalis m		600	5,120
Corynebacterium diphtheriae 300*		40	>81,920
Streptococcus viridans "lab"*		20	>81,920
Salmonella paratyphi		<20	40
Shigella paradysenteriae		<20	40
Penicillin-resistant staphylococci, No. 1	<20	640	5,120
" " " 2	<20	320	5,120

* Serum incorporated in assay medium.

Toxicity Studies

The effects of a crude neutral fraction and Pyo II were tested on protozoa. These preparations, which were dissolved in alcohol, precipitated upon dilution with the culture medium unless the concentration was kept low. For this reason, concentrations ranging from 0.1 to 5.0 γ per ml. were used. At these levels no effects were observed on *Paramecium caudatum*, *Euglena viridis*, and *Amoeba proteus*.

The toxicity of a crude neutral fraction containing approximately 1000 "50 per cent acid inhibition" units per mg. was tested in adult mice. No ill effects were observed after single subcutaneous injections of 5.5 mg. in benne oil or intravenous injection of 10 mg. in benne oil in four doses during 48 hours. When a benne oil solution containing 11.5 mg. of the crude extract was given orally in a single dose to a mouse, no observable effects were

noted. Likewise a total of 27.5 mg. in benne oil given orally in eleven doses over 7 days produced no ill effects. Finally 100 mg., dissolved in cooking fat (Spry), were given orally without harm to a mouse.

A single oral dose of 25 mg. suspended in a starch-gelatin solution or a total of 37 mg., in the same medium, given over a period of 8 days, caused no ill effects.

The intestinal contents of the mice which had received Pyo for 2 to 8 days were examined for protozoa. No noteworthy difference could be observed between experimental and control animals.

SUMMARY

Methods are described for the production, purification, and isolation of some new antibiotic substances elaborated by *Pseudomonas aeruginosa.*

Analytical data, the physical and chemical properties of these pure crystalline materials, and some of their derivatives are presented. Evidence is presented which indicates that at least four of our substances (Pyo Ib, Pyo Ic, Pyo II, and Pyo III) are structurally related.

In general, the Pyo compounds are active against Gram-positive but considerably less active against Gram-negative organisms.

Preliminary toxicity studies indicate that these substances in the crude and in the pure state are non-toxic in the animal organism.

We are indebted to the Theelin Fund administered by the Committee on Grants for Research of St. Louis University for financial support, to Dr. Robert D. Coghill for a sample of crystalline sodium salt of penicillin G and for a comparison of the antibiotic activities of penicillin and Pyo II by the Oxford method, and to Mrs. E. A. Doisy and Mr. Joseph Yglesias for their helpful technical assistance.

BIBLIOGRAPHY

1. Pasteur, L., and Joubert, J., *Compt. rend. Acad.*, **85**, 101 (1877).
2. Emmerich, R., *Arch. Hyg.*, **6**, 442 (1887).
3. Bouchard, C., *Compt. rend. Acad.*, **108**, 713 (1889).
4. Woodhead, G. S., and Wood, C., *Compt. rend. Acad.*, **109**, 985 (1889).
5. Charrin, M., and Guignard, L., *Compt. rend. Acad.*, **108**, 764 (1889).
6. Blagovestchensky, N., *Ann. Inst. Pasteur*, **4**, 689 (1890).
7. von Freudenreich, E., *Ann. Inst. Pasteur*, **2**, 200 (1888).
8. Emmerich, R., and Löw, O., *Z. Hyg. u. Infektionskr.*, **31**, 1 (1899).
9. Klimoff, J., *Z. Hyg. u. Infektionskr.*, **37**, 120 (1901).
10. Dietrich, A., *Centr. Bakt.*, **31**, 165 (1902).
11. Raubitschek, H., and Russ, V. K., *Centr. Bakt.*, **48**, 114 (1909).
12. Fukuhara, Y., *Z. Immunitätsforsch. u. exp. Therap.*, **9**, 75 (1911).

13. Ohkubo, S., *Z. Immunitätsforsch. u. exp. Therap.*, **5**, 428 (1910).
14. Bocchia, I., *Centr. Bakt.*, **50**, 220 (1909).
15. Wagner, W., *Z. Immunitätsforsch. u. exp. Therap.*, **63**, 483 (1929).
16. Kramer, H., *Z. Immunitätsforsch. u. exp. Therap.*, **84**, 505 (1935).
17. Hosoya, S., *Compt. rend. Soc. biol.*, **99**, 771 (1928).
18. Gundel, M., and Wagner, W., *Z. Immunitätsforsch. u. exp. Therap.*, **69**, 63 (1930-31).
19. Hettche, H. O., *Arch. Hyg.*, **107**, 337 (1932).
20. Hettche, H. O., *Klin. Wochschr.*, **12**, 1804 (1933).
21. Hettche, H. O., *Z. Immunitätsforsch. u. exp. Therap.*, **83**, 499 (1934).
22. Hettche, H. O., *Z. Immunitätsforsch. u. exp. Therap.*, **83**, 506 (1934).
23. Hettche, H. O., and Vogel, W., *Arch. Hyg.*, **117**, 234 (1937).
24. Birch-Hirschfeld, L., *Z. Hyg. u. Infektionskr.*, **116**, 304 (1934-35).
25. Schoental, R., *Brit. J. Exp. Path.*, **22**, 137 (1941).
26. Wrede, F., and Strack, E., *Z. physiol. Chem.*, **140**, 1 (1924).
27. Wrede, F., and Strack, E., *Z. physiol. Chem.*, **177**, 177 (1928).
28. Wrede, F., and Strack, E., *Z. physiol. Chem.*, **181**, 58 (1929).
29. Lartigau, A. J., *J. Exp. Med.*, **3**, 595 (1898).
30. Hadley, P., *J. Infect. Dis.*, **40**, 1 (1927).
31. Yasuda, M., *J. Biol. Chem.*, **94**, 401 (1931-32).
32. Brady, J., *J. Chem. Soc.*, 756 (1931).

LETTERS TO THE EDITORS

CRYSTALLIZATION OF MEXICAIN

Sirs:

In the latex from *Pileus mexicanus*, Castañeda-Agulló *et al.*[1] found a highly active proteolytic enzyme which was called mexicain and which has now been crystallized.

2 volumes of water were added to 1 volume of fresh latex and the pH adjusted to 7.5 with 0.5 N sodium hydroxide with constant stirring. The mixture is placed in a refrigerator at 5° for 24 hours. The solution is then filtered through Hyflo Super-Cel and the pH adjusted to 5.5 with 0.05 N hydrochloric acid. At this pH the solution has an opalescent cast and after 24 hours at 5° there appears a fine precipitate of homogeneous crystals in the form of lanceolate plaques.

In three successive crystallizations the same crystalline form always appeared. These crystals have a protein reaction and have an activity in digestion of proteins and milk clotting 4- to 5-fold that of dry latex *in vacuo*.

The crystalline enzyme is very soluble and its solutions, at pH 5.8, will not lose their activity at room temperature and do not need activators, such as sodium cyanide or cysteine, for stability of enzymatic power.

In a sample of fresh latex collected in May, 1944, and stored in a refrigerator for 1 year, at 5°, identical active crystals were obtained by this same method, without chemical activators, but, however, with less activity than those obtained from latex collected more recently.

Laboratorios de Fisiología
Instituto Politécnico Nacional
Departamento de Fisiología de la Escuela de Medicina
Universidad Nacional
México, D. F.

M. Castañeda-Agulló
A. Hernández
F. Loaeza
W. Salazar

Received for publication, June 2, 1945

[1] Castañeda, M., Gavarron, F. F., and Balcazar, M. R., *Science*, **96**, 365 (1942).

OBSERVATIONS ON THE GROWTH-STIMULATING ACTION OF CERTAIN PROTEINS ADDED TO PROTEIN-FREE DIETS COMPOUNDED WITH AMINO ACIDS*

Sirs:

For several years it has been recognized that rats, mice, or chicks grow less rapidly when a mixture of amino acids is used as the N source in the diet than when casein is employed. Opinions about why this should be so have differed. Therefore, the following new facts and interpretations are presented to aid in the understanding of the phenomenon.

Mice grew at a suboptimal rate when the casein used as the N source in their diet was hydrolyzed with acid, and the hydrolysate was fortified with cystine and tryptophane. This was demonstrated by feeding young mice a diet composed of sulfuric acid-hydrolyzed casein, prepared as described previously,[1] 18 parts, *dl*-tryptophane 0.8, *l*-cystine 0.3, sucrose 75, salts[2] 5, fortified corn oil 1, and the crystalline vitamins as recommended earlier.[3] To render the ration less hygroscopic, 10 parts of cellulose were added.

Small amounts of intact casein and even smaller amounts of crystalline trypsinogen when added to the above basal ration caused restoration of normal growth. On the other hand, coagulated egg white was without beneficial effect. The data show further that a concentrate of strepogenin[4] prepared from a tryptic digest of casein and fed at a level equivalent in strepogenin content to the minimal effective dose of casein was approximately as active as this protein. Finally, when the strepogenin potency of the concentrate was destroyed by acid hydrolysis, growth-promoting power was lost.

Strepogenin is an unidentified peptide-like bacterial growth factor[5] which has recently been shown to be a constituent of several pure proteins.[4] It has been found abundantly in casein, but only in traces in dialyzed egg white. Crystalline trypsinogen is apparently a much richer source than casein. Strepogenin is destroyed by hydrolysis with strong acid.

These properties correlate sufficiently well with the present findings with mice to suggest that the growth-promoting powers of some proteins added to diets based on amino acids may be attributed to strepogenin. Indeed,

* With the technical assistance of M. L. Collyer and B. Bailey.

[1] Woolley, D. W., *J. Biol. Chem.*, **140**, 453 (1941).

[2] Phillips, P. H., and Hart, E. B., *J. Biol. Chem.*, **109**, 657 (1935).

[3] Woolley, D. W., *J. Biol. Chem.*, **154**, 31 (1944).

[4] Woolley, D. W., and Sprince, H., *Federation Proc.*, **4**, 164 (1945). Sprince, H., and Woolley, D. W., *J. Am. Chem. Soc.*, in press.

[5] Sprince, H., and Woolley, D. W., *J. Exp. Med.*, **80**, 213 (1944).

Growth Response of Mice Fed a Casein Hydrolysate Ration

Addition to basal ration		No. of animals	Average change in weight
	per cent		*gm. per wk.*
None		12	1.7
Casein (vitamin-free)	10	4	3.3
	2	4	3.1
	1	7	2.4
Dialyzed heated egg white	5	7	2.1
Denatured crystalline trypsinogen	0.5	4	3.6
Strepogenin concentrate	Equivalent to 2% casein	8	3.0
Hydrolyzed strepogenin concentrate	" " 4% "	4	1.3

the present experiments were designed and undertaken in an effort to learn whether this bacterial growth factor was of importance in animal nutrition. This question cannot be answered until pure strepogenin is available, but the data show that proteins rich in this factor (casein and trypsinogen) have growth-promoting powers not possessed by proteinaceous material low in strepogenin (egg white). Egg white is not deficient in the known amino acids. Thus, for mice as well as for bacteria,[4] the nutritional properties of proteins are not related solely to the amino acids obtainable by acid hydrolysis.

The Rockefeller Institute for Medical Research
New York

D. W. WOOLLEY

Received for publication, July 6, 1945

SYNTHESIS OF LABILE METHYL GROUPS IN THE WHITE RAT

Sirs:

In the first report of growth experiments with homocystine diets supplemented with choline, du Vigneaud and coworkers[1] called attention to the fact that occasionally they encountered animals "capable of showing some growth on the homocystine diet without added choline." It was suggested[1] that labile methyl groups had been synthesized by the intestinal bacteria of these rats. Growth of rats receiving methyl-free, homocystine diets has also been reported recently by Bennett, Medes, and Toennies.[2]

In the present communication, we wish to report evidence that indicates the synthesis of a small amount of methyl groups in the white rat maintained on a diet adequate in labile methyl groups. This evidence was obtained by maintaining the deuterium concentration of the body water of rats at about 3 atom per cent and subsequently isolating the choline from the tissues of these rats. If, under these conditions, deuterium was found in the methyl groups of choline, synthesis of the methyl group must have taken place somewhere in the animal, either in the tissues or in the intestinal tract. It was extremely unlikely that a direct exchange reaction would bring about the appearance of deuterium in the methyl groups under these conditions.[3]

Two rats were fed a casein diet having the following percentage composition: vitamin-free casein 20, salt mixture[4] 4, Crisco 19, sucrose 56, corn oil containing fat-soluble vitamins[5] 1. Each rat received daily 0.20 mg. of *dl*-calcium pantothenate, 2 mg. of *p*-aminobenzoic acid, 5 mg. of inositol, and 0.02 mg. each of thiamine chloride, riboflavin, nicotinic acid, and pyridoxine hydrochloride. The deuterium concentration of the body water was raised to approximately 3 atom per cent by the intraperitoneal injection of the necessary amount of 99.5 per cent D_2O and was maintained by providing drinking water containing 4 atom per cent D_2O for 3 weeks. The deuterium contents of the tissue choline, isolated as choline chloroplatinate,[3] and of trimethylamine prepared from it[3] are given in the table.

[1] du Vigneaud, V., Chandler, J. P., Moyer, A. W., and Keppel, D. M., *Proc. Am. Soc. Biol. Chem.*, *J. Biol. Chem.*, **128**, p. cviii (1939); **131**, 57 (1939).

[2] Bennett, M. A., Medes, G., and Toennies, G., *Growth*, **8**, 59 (1944).

[3] du Vigneaud, V., Cohn, M., Chandler, J. P., Schenck, J. R., and Simmonds, S., *J. Biol. Chem.*, **140**, 625 (1941).

[4] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **37**, 572 (1919).

[5] du Vigneaud, V., Kilmer, G. W., Rachele, J. R., and Cohn, M., *J. Biol. Chem.*, **155**, 645 (1944).

Deuterium Content of Methyl Groups of Choline

Change in body weight	Average deuterium in body water (*A*)	Deuterium content			Per cent of choline methyl derived from body water $\left(\frac{B}{A} \times 100\right)$
		Choline chloroplatinate	Trimethylamine chloroplatinate	Methyl group of choline (*B*)	
gm.	*atom per cent*	*atom per cent*	*atom per cent*	*atom per cent*	
102–145	3.1	0.60 ± 0.01*	0.15 ± 0.06		
			0.21 ± 0.01*	0.24†	7.7†
116–165	3.2	0.39 ± 0.06	0.23 ± 0.08		
			0.25 ± 0.02*	0.27†	8.5†

* Micro deuterium method with the mass spectrometer.

† Value calculated from the data obtained by the micro deuterium method because of the smaller error in analysis.

These results indicate a small, but not insignificant, amount of labile methyl group synthesis. The present data do not distinguish between direct synthesis by the tissues and synthesis by intestinal bacteria with subsequent utilization of the methyl groups in the tissues. On the basis of the facts we now have concerning the labile methyl groups in the diet and in metabolism, we feel that the latter explanation involving intestinal bacteria is the most logical interpretation of our results.

The authors wish to thank Dr. D. Rittenberg of the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, for micro deuterium analyses carried out by an unpublished method.

Department of Biochemistry
Cornell University Medical College
New York City

VINCENT DU VIGNEAUD
SOFIA SIMMONDS
JOSEPH P. CHANDLER
MILDRED COHN

Received for publication, June 28, 1945

INDEX TO AUTHORS

INDEX TO SUBJECTS